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THE TOXICITY OF VICTORIA BLUE 4 R. FOR B. PARATYPHOSUS A, B. PARATYPHOSUS B AND B. ENTERITIDIS

OSCAR TEAGUE

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While studying three years ago the relative toxicity of various dyes on strains of *B. typhi*, *B. paratyphi* A and B and *B. coli*, I noticed a marked difference in the toxicity of Victoria blue 4 R. for *B. paratyphi* A and *B. paratyphi* B. A careful comparison of the growth of a fairly large number of strains of these organisms and of *B. enteritidis*, upon the same lot of agar containing different amounts of the stain, was made and, though the results were clearly defined, viz., the paratyphoid B bacillus being more readily inhibited than paratyphoid A and *B. enteritidis*, yet they did not seem at the time of sufficient importance to warrant their publication.

At present, however, as infections with these organisms have become more common, and a study of their characteristics has been undertaken in a number of American laboratories, it would seem that my observations might possibly supplement some of these studies and should, therefore, be presented.

Meat infusion agar was prepared in the usual way with 1 per cent Witte's peptone and 1/2 per cent sodium chloride in the Arnold sterilizer. It was cleared with egg white, filtered through cotton and titrated to + 1. The medium was sterilized by being heated for three successive days in the Arnold. A stock solution of Victoria blue 4 R. was prepared and the requisite amounts of this were added to comparatively large amounts of the agar to yield 1/20, 1/30, 1/40 and 1/50 per cent respectively of the stains; the required number of plates were poured from each. Hence all the plates containing a given per cent of the stain were uniform in every respect.

For the tests fresh transplants of the cultures[1] were used after twenty-four hours' incubation. Suspensions of the different cultures in saline solution were prepared of approximately the same cloudiness, and 1 loop of an appropriate dilution of each was inoculated upon plates with different percentages of the Victoria blue and upon a control plate containing no stain. The cultures were emulsified and inoculated in lots of four in the order recorded in table 1.

All of the cultures were subjected to the test on the same day, in order to ensure uniformity of conditions. It was not convenient to count the colonies on all the plates at the end of twenty-four hours, so the number was recorded as "numerous" where there was a good growth with obviously little or no inhibition due to the presence of the dye Eighteen cultures of *B. paratyphi* A., 18 of *B. paratyphi* B and 11 cultures of *B. enteritidis* were employed. Some of these may have been duplicates, having been obtained by different laboratories from the same original strain. The paratyphoid A strains without exception gave good growth even on the plates containing the maximum amount of the stain; the colonies were quite large after twenty-four hours' incubation and the reduction in number as compared with the control plates was usually considerably less than 50 per cent. The paratyphoid B cultures, on the other hand, were almost completely inhibited in their growth by 1/20 per cent and 1/30 per cent of Victoria blue and some of them were inhibited also by 1/40 per cent and 1/50 per cent of the dye; the few colonies that did appear were usually quite small after twenty-four hours' incubation. The *B. enteritidis* culture behaved on the plates like the paratyphoid A cultures. This seemed surprising, as they resemble paratyphoid B more closely in most of their characteristics than paratyphoid A.

[1] Stock laboratory cultures of *B. paratyphi A* and *B* and of *B. enteritidis* were obtained from the New York City Health Department Research Laboratory, the Hygienic Laboratory and the United States Army Medical School in Washington, the Mulford Laboratories, the Museum of Natural History, New York, and from the Department of Tropical Medicine at Harvard.

TABLE 1

Victoria blue 4 R

CULTURES	1/20 PER CENT		1/30 PER CENT		1/40 PER CENT		1/50 PER CENT		CONTROL
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	
B N. Y. Hosp...	0	0	0	3	4	45	0	81	246
B 97............	0	0	0	2	0	13	0	14	92
A 60............	f. num.	121	f. num.		f. num.		f. num.		132
B 25............	0	0	0	3	3	11	3	8	174
B And..........	0	0	0	0	0	0	0	8	271
B 66............	0	0	0	0	0	0	0	5	119
A 57............	f. num.	106	f. num.	118	f. num.	92	f. num.	117	218
Gärtner Mt. S...	num.	189	num.		num.		num.		320
A 3.............	f. num.	109	num.		num.		num.		156
B Am...........	0	7	few	46	f. num.	49	num.	100	111
A 56............	num.	197	num.		num.		num.		310
A 63............	num.	68	num.	67	num.		num.	76	140
B 1.............	0	0	0	0	0	1	0	1	105
B 59............	1	7	10	12	20	25	18	23	152
A And..........	num.	60	num.	55	num.	73	num.		92
Schottmüller....	0	0	0	3	few	27	f. num.	37	100
B 52............	0	0	0	0	0	1	0	2	137
Gärtner And.....	num.	145	num.	171	num.		num.		169
B 2.............	0	0	0	0	0	0	0	1	74
Kurth..........	0	0	0	3	few	21	few	49	103
A 55............	num.	448	num.	58	num.	48	num.	55	81
A 4.............	num.	59	num.	97	num.	92	num.	86	112
B 65............	0	0	0	0	0	1	1	5	101
B Boston 1......	0	0	0	1	few	34	28	40	156
A 54............	157		num.		num.		num.		186
A 62............	29	29	30	33	28	28	32	32	75
A 7.............	num.	97	num.		num.		76		146
A 1.............	num.	169	num.		num.		num.		222
B Boston 2......	3	3	18	18	num.	96	f. num.		175
Enteritidis 69...	num.	260	num.		num.		num.		400
B 61............	0	3	f. num.	65	num.	76	num.	112	150
A 58............	num.	85	num.	90	num.	96	num.		150

TABLE 1—Continued

CULTURES	1/20 PER CENT		1/30 PER CENT		1/40 PER CENT		1/50 PER CENT		CONTROL
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	
A Yale..........	num.	185	num.		num.		num.		300
Paratyphoid A..	num.	100	num.		num.		num.		174
B 53............	0	13	f. num.	43	num.	66	num.	66	81
B Roanoke......	few	20	f. num.	34	num.	51	num.	62	50
Enteritidis 14....	num.	117	num.		num.		num.		124
A 2..............	num.	67	num.	65	num.	87	num.	94	86
Gärtner 93.......	num.	71	num.	103	num.		num.		119
A Cafirin........	num.	129	num.	104	num.		num.		140
A W. B..........	num.	110	num.		num.		num.		131
Enteritidis 70....	num.	192	num.		num.		num.		161
Enteritidis And..	few	31	few	37	f. num.	66	f. num.	56	127
Enteritidis 47....	num.	160	num.		num.		num.		160
Enteritidis 67....	num.	148	num.		num.		num.		230
Enteritidis 64....	f. num.	100	num.		f. num.		num.		206

num. = numerous colonies.
f. num. = fairly numerous colonies.

Five of the paratyphoid B. cultures (Am. Boston 2, 61, 53, and Roanoke) and 2 of the B. enteritidis cultures (And. and Whit.) gave intermediate results; that is, they were not inhibited as completely as paratyphoid B cultures, nor did they give as good growth as the paratyphoid A cultures. The paratyphoid organisms are known to undergo mutations occasionally after having been grown on artificial culture media for long periods of time, the B type assuming characteristics of the A type and vice versa. It seemed not unlikely that these few cultures may have behaved atypically with regard to growth on the Victoria blue agar because such mutations had occurred in them. Another possibility is that they may have had their source from some of the lower animals and not from infected human beings.

Similar experiments were performed with cultures of *B. suipestifer*, *B. typhi murium* and *B. Danysz*, the results of which are recorded in table 2.

TABLE 2

Victoria blue 4 R

CULTURE	1/20 PER CENT		1/30 PER CENT		1/40 PER CENT		CONTROL
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	
Typhi murium 0231...	8	26	few	68	few	91	234
Hog cholera 052......	1	4	few	15	2	6	250
Danysz B............	num.	113	num.	num.	num.	num.	151
Guinea pig Paratyph. B 34...............	0	0	0	0	0	0	159
Hog cholera Ithaca...	f. num.	130	num.	num.	f. num.	num.	274
Danysz 540...........	num.	198	num.	num.	num.	num.	231
Typhi murium 15.....	num.	176	num.	num.	num.	num.	250
Cholera suis C........	0	3		4			238
Danysz A............	num.	151	num.	158			290
Danysz 590...........	num.	158	num.	num.	num.	num.	214
Hog cholera 2228.....	f. num.	105	f. num.	f. num.	f. num.	96	?
Hog cholera Page 118.	0	0	0	2	0	0	257
Suipestifer 332........	12	20			few	48	233
Typhi murium 237....	num.	102			num.	92	130
Suipestifer 553........	num.	300			num.	num.	412
Typhi murium 0299...			0	0	0	0	158
Hog cholera 3890.....			num.	182	num.	101	352
Typhi murium 0301...	num.	102	num.	num.			147
Hog cholera 138......	0	0	0	12			366
Suipestifer 258........	few	40	f. num.	72	f. num.	74	339
Typhi murium 0300...	num.	240	num.	num.	num.	num.	325
Hog cholera 048......	f. num.	110	f. num.	num.	num.	num.	180
Suipestifer 231........	num.	192	num.	num.	num.	num.	202
Hog cholera 053......	few	57	f. num.	84	f. num.	96	120
Typhi murium 0229...	num.	125	num.	num.	num.	num.	163
Hog cholera 051......	0	0	0		0	0	134
Hog cholera 050......	num.	133	num.	num.	num.	num.	124
Hog cholera 049......	few	90	few	127	f. num.	141	219
Rabbit paratyph. B 45			3	8	2	6	365

For the sake of clearness the results of these experiments are summarized in table 3.

It is seen that all of the *B. Danysz* cultures and 5 of the 7 *B.*

TABLE 3

	NUMBER OF STRAINS SHOWING ON VICTORIA BLUE 4R. AGAR		
	Marked inhibition	Moderate inhibition	No inhibition
B. suipestifer	6	1	9
B. typhi murium	1	1	5
B. Danysz	0	0	4

typhi murium cultures showed good growth on Victoria blue agar. The hog cholera organisms fall into two sharply defined groups; 6 of the cultures are almost completely inhibited, like paratyphoid B strains, 9 strains are not inhibited and only one strain shows moderate inhibition. Jordan (1917) says:

A few strains of porcine origin possess the characteristics of the *B. paratyphosus B.* type. These, however, are all strains that have been under cultivation for some time. A number of strains, particularly some of the older cultures, have shown marked variations since they came into my hands. Some of the difficulty experienced by previous observers in the differentiation of the *B. paratyphosus* and *B. suipestifer* types has been probably due to the existence of stock cultures labelled in one way or the other, but possessing the cultural and agglutinative character of the opposite type. The extent to which transformation of one type into the other occurs under the ordinary conditions of laboratory cultivation is a matter for further investigation.

In view of this situation with regard to the *B. suipestifer* cultures, one is tempted to conclude that paratyphoid B, alone of all the paratyphoid enteritidis group of organisms is inhibited in its growth by the strength of Victoria blue 4 R. employed in the above experiments. It would seem highly desirable to repeat the experiments with freshly isolated strains.

REFERENCE

Jordan, E. O. 1917 Jour. Inf. Dis., 20, 477.

AUTOMATIC WATER LEVEL FOR ARNOLD STERILIZERS

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Experienced technicians, and untrained students, alike err occasionally in allowing Arnold sterilizers to burn dry, involving rapid depreciation of the apparatus and inconvenience incidental to the necessity of repairs.

The following arrangement has been in operation for some time in our laboratory where over one hundred and seventy-five different persons use the group of twelve connected sterilizers with absolutely no required attention on their part to the water supply.

The water main is connected to the flush tank of a water closet, placed on the bench at the end of the battery of sterilizers. The inlet valve is operated by the rise and fall of the usual brass globe, so that a constant level is maintained. In case any defect should cause the valve to fail to close, an overflow is arranged to carry the surplus water to the sewer, thus avoiding the possibility of a flooded room. This contingency has not yet arisen. The sterilizers which stand upon gas plates are connected with the supply tank and with one another by means of siphons made of $\frac{3}{8}$ inch lead pipe bent into U tubes. These are started by filling with water and holding the ends closed with the fingers until one end can be released under the water in the tank. Proceeding thus from the tank to the first sterilizer, and from this to the second, the successful operation of the connecting siphons was established to start with one at a time.

The water, of course, soon reaches a common level in all, as determined by the setting of the float valve. Any operation of the sterilizers draws upon the supply tank through the siphons for water sufficient to maintain the common level, and the lowering of the float operates the valve automatically to replenish the supply as needed.

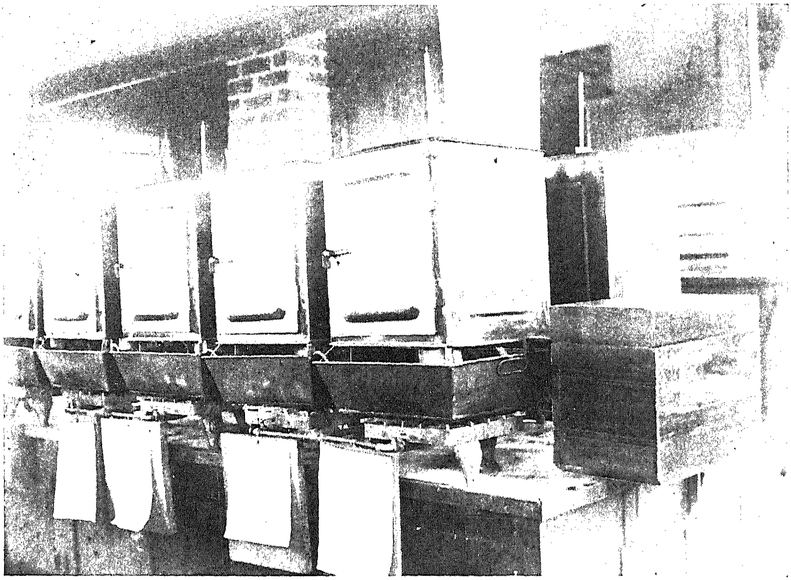

FIG. 1. AUTOMATIC WATER LEVEL FOR ARNOLD STERILIZERS, SHOWING METHOD OF ASSEMBLY WITH CONNECTING SIPHONS

AN AEROBIC SPORE-FORMING BACILLUS GIVING GAS IN LACTOSE BROTH ISOLATED IN ROUTINE WATER EXAMINATION

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In the course of routine water examinations at this station, the writer has, on several occasions, isolated aerobic lactose-fermenting organisms which have been demonstrated to be spore-forming. So far as can be ascertained by a fairly complete review of the published literature, there has been no such organism previously described.[1] In water work two large groups of bacteria generating gas from lactose are recognized. The first are the aerobic non-spore-forming bacilli of the coli-aerogenes group, and the second the anaerobic spore-forming bacilli of the sporogenes group. The significance of the presence in a water of members of either of these groups has been pretty well established. The organism to be described lies midway between these two groups, in that it is aerobic as well as spore-forming. Just what its sanitary significance is, remains to be established.

ISOLATION

It has been the routine procedure in this laboratory to isolate for study one culture of *B. coli* from each sample of water examined. This is effected by carefully fishing one isolated colony

[1] Since this article went to press, the writer has come across an article on systematic bacteriology of water by S. De M. Gage and E. B. Phelps in Am. Pub. Health Ass. Rep. 1902, **28**, 402, 412. In the table given in this article a few cultures are noted as being aerobic lactose fermenters. In the opinion of Mr. Gage it is very unlikely that the organism now being described could be among those described by him.

from the confirmatory Endo's medium plate, made from the lactose broth tube inoculated with the water sample. The colony is inoculated into lactose broth, to confirm gas production, and if this tube is positive the organism is again plated on Endo's medium to determine if it be pure. If this plate reveals the presence of but one form, a colony is fished to an agar slant. A smear from the culture on the slant, after forty-eight hours' growth at 37°C., is stained by Gram's method, and if only Gram-negative, non-spore-forming bacilli are seen, the culture is considered pure and made up of *B. coli*.

Following the above outlined procedure, eight cultures from one source[2] were found which gave gas in lactose broth, but which on the agar slant grew very differently from *B. coli*. They appeared on staining to be large Gram-negative, fusiform rods containing spores, together with large Gram-negative vegetative bacilli. For some time the writer was under the impression, gained through previous experience, that this spore-bearer was a contamination form and that the gas production would finally be found to be due to *B. coli*. Accordingly, attempts were made to purify the culture by plating on Endo's medium, agar and gelatine. The colonies on all of the media appeared to contain but one organism. When transferred to agar slants the growth was macroscopically and microscopically like the agar slant culture first alluded to. That the gas production was due to the spore-forming organism was definitely proved by the heat-resistance experiments next performed.

RESISTANCE TO HEAT

The organism will live and generate gas in lactose broth after being subjected to 95°C. for twenty minutes or to boiling water temperature for ten minutes, but is killed if subjected to the latter temperature for fifteen minutes. The writer has been checked in these results by another bacteriologist in this labora-

[2] Since the writing of this article the organism has been isolated three additional times—once from the tap water of Covington, Ky., and twice from raw tannery trade wastes. These three cultures resemble the eight previously isolated in all particulars.

tory, working independently. The tests were run as follows: tubes of standard lactose broth, with inverted vials, were placed in a water-bath, brought to the desired temperature and regulated to within 1°C. After the tubes had attained the temperature of the water-bath, 0.1 cc. of a light suspension of the organism taken from the surface of a seventy-two hour old agar slant was carefully added to the tube and the time noted. At suitable intervals, two tubes were withdrawn and put into cold water to cool. After seventy-two hours' incubation at 37°C. the tubes were examined and the growth and gas production recorded. Where there was no gas production the tubes were sterile. Agar slant cultures made from the broth tubes showing gas revealed the spore-former in pure culture.

MORPHOLOGY AND STAINING CHARACTERISTICS

In smear preparations made from forty-eight hour old agar slants three distinct forms, representing different stages of the bacillus, are seen. First there is the vegetative cell, a regular rod with rounded ends, Gram negative, 4.5 to 5.5 by 0.8 to 1 μ Next is the spore-bearer, spindle-shaped, Gram-negative, about 3 by 1.5 μ. With ordinary stains the spore is shown as a central, oval, unstained area, and with Müller's spore stain is readily demonstrated. The third form is the free spore, oval, regular, 2 by 1.5 μ, showing deep red with the spore stain. These occur most frequently and regularly in older cultures.

No capsules could be demonstrated by the use of the Welch capsule stain in smear preparations from milk cultures. When stained by Löffler's method, the organisms show numerous peritrichic flagella, some bacilli showing as many as 16 to 18. Notwithstanding this fact, motility has not been observed when using the hanging drop method.

APPEARANCE AND REACTIONS IN VARIOUS MEDIA

Agar slant. Growth quite distinctive. At 37°C., in twenty-four hours, thin transparent veil-like growth over entire surface except the very top. Growth lobate along upper edge. Micro-

scopically—in twenty-four hours mainly vegetative forms, in forty-eight hours spore-bearing forms and later only free spores.

Agar stab at 37°C. Growth along entire line of inoculation into depths of agar. Growth somewhat echinulate. In meat infusion agar many gas bubbles, due to fermentation of muscle sugar (inosite).

Agar plate. Nutrient agar twenty-four hours at 37°C., colonies discrete, round, thin and small (1 mm. diameter), edges smooth.

Endo's plates at 37°C. Twenty-four hours, colonies pink with red center, irregular contour, 1 to 2 mm. diameter, little or no sheen. Colonies forty-eight hours, deep red, much sheen in colonies and surrounding medium. Latter point distinctive.

Gelatin plate at 20°C. Forty-eight hours, colonies small (0.5 to 1 mm. diameter), round, *incipient* liquefaction. Colonies seventy-two hours—liquefaction, round, edges regular, 2 to 3 mm. diameter.

Gelatin stab at 20°C. Forty-eight hours, beginning liquefaction; in seventy-two hours liquefaction infundibuliform, slight precipitate.

Carbohydrates. In standard extract broth to which has been added one percent of the following carbohydrates, acid and gas are formed: (1) Glucose, (2) laevulose, (3) raffinose, (4) maltose, (5) sucrose, (6) lactose, (7) inulin, (8) starch, (9) glycerol, (10) mannitol. No acid or gas and little growth in dulcite broth, which remained clear and limpid. In other broths gas usually appeared in twenty-four hours. Media uniformly clouded, slight stringy precipitate, no pellicle. Media forty-eight hours, slightly viscous.

Glucose neutral-red broth. Same reaction as that of *B. coli*, i.e., yellow fluorescence with gas formation.

Clark's glucose, peptone, phosphate, medium (0.5 per cent glucose, 0.5 per cent K_2HPO_4 and 0.5 per cent peptone broth), typical reaction of "Grain" type coli, in forty-eight hours at 37°C., i.e. reaction alkaline to methyl red, Voges-Proskauer test positive.

Limiting hydrogen-iron concentrations. Clark's glucose peptone-phosphate medium was used after being adjusted with NaOH or HCl to various hydrogen-ion concentrations. Growth between $P_H = 5.0$ to $P_H = 9.0$ inclusive. In tube $P_H = 9.0$ very scanty growth and only bubble of gas. In all other tubes much gas, and growth luxuriant.

Indol production in 1 per cent peptone, four days at 37°C. No indol detected when tested for by the nitrite and by Ehrlich's para-dimethyl-amido-benzaldehyde method.

Reduction of nitrates. In 0.1 per cent peptone + 0.02 per cent $NaNO_3$ solution, four days at 37°C., no reduction to nitrites.

Litmus milk at 37°C. In twenty-four hours acid, in forty-eight hours partially reduced, coagulated with extrusion of whey, beginning digestion of curd.

Lactose bile at 37°C. Ninety-six hours, no gas or growth.

Chromogenesis. None noted on any media used.

OCCURRENCE AND SIGNIFICANCE

Dr. J. S. Bolten, working in this laboratory, isolated what the writer believes to have been the same or a similar organism from a sample of sewage which had been taken from Mill Creek during the winter of 1916 and stored for forty-one days. Reference to his unpublished notes which are on file at this station, shows that the organism had morphological and cultural characteristics similar to those detailed above. Owing to rather variable and uncertain heat resistance experiments, which Dr. Bolten deemed inconclusive, he was unable to state definitely that the spore-bearer was the gas-former. However, Dr. Bolten used for seeding material in the heat resistance experiments, cultures in lactose broth, in which medium, sporulation is indefinite and delayed.

Regular examinations are made in this laboratory of samples of water from various sources. These include the Ohio River and its tributaries in the vicinity of Cincinnati, and the tap supplies of Cincinnati and two Kentucky cities on the Ohio River opposite this city. The organism herein described has

been isolated from but one of these sources—the water supply of Newport, Kentucky. Up to date it has been obtained from the samples collected on the following eight days: January 30, February 8, 10 and 26, March 31, and April 2, 11, and 20, 1917. Newport, Kentucky, uses Ohio River water after subjecting it to treatment and storage. Treatment consists of addition of small amounts of calcium hypochlorite, and of lime and iron, and the total storage is estimated at twenty days.

This organism during the times of its occurrence might cause considerable error in the determination of the colon index. During the months of January to April, 1917, inclusive, of 40 aerobic gas formers isolated from 91 samples of Newport tap water, 32 or 80 per cent were of the coli-aerogenes group while 8 or 20 per cent were this spore-former. Due to its rarity and limited occurrence, however, it could not constitute a source of appreciable error in routine water examinations in most localities. Over 17,000 samples have been examined at this station within the past three years, and this spore-former has been isolated but eight times and, as detailed above, from but one source. Other water workers have never reported its occurrence, and it is likely that in most waters it is exceedingly rare, if not entirely absent.

SUMMARY

An aerobic bacillus, giving gas from lactose, and demonstrated to be spore-forming, has been isolated eight times between January 30 and April 20, 1917, from the tap water of Newport, Kentucky. This organism is believed to be a species whose isolation has never before been described.

The writer wishes to acknowledge his indebtedness to Surgeon W. H. Frost, under whose direction this study has been made.

A SIMPLE METHOD FOR THE CLASSIFICATION OF BACTERIA AS TO DIASTASE PRODUCTION

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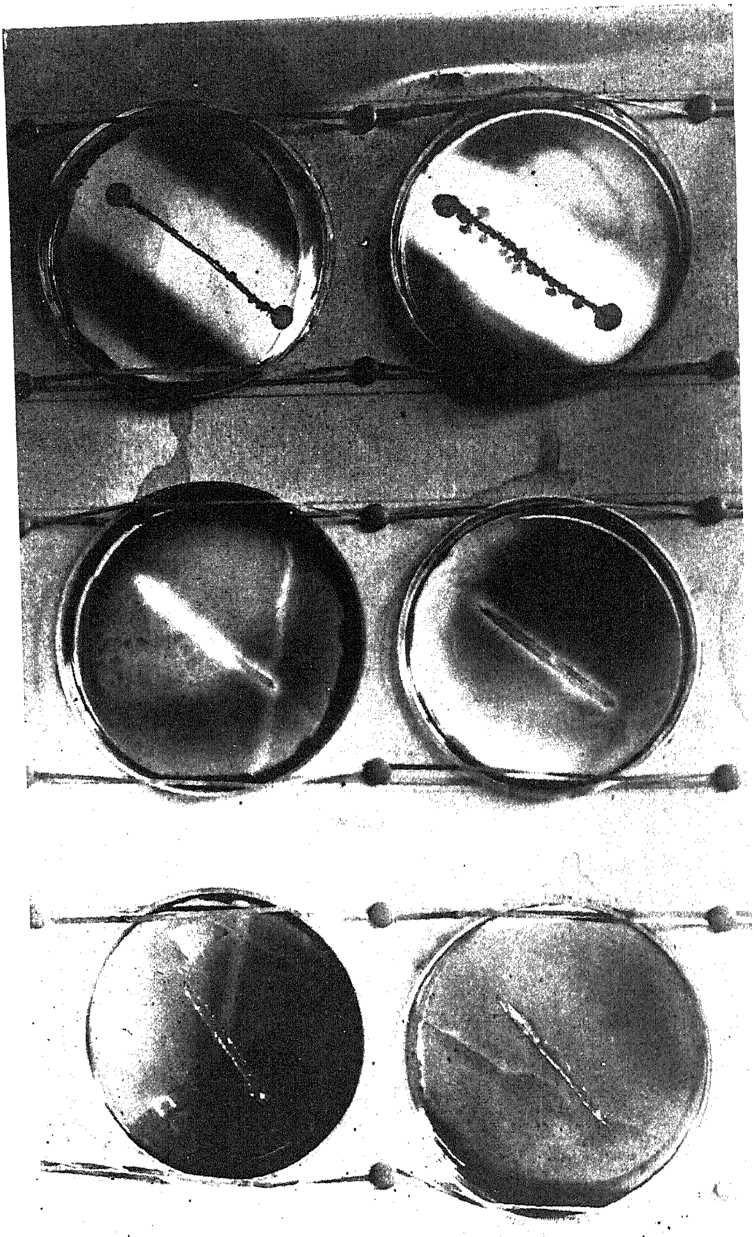
Among the various physiological reactions selected by the Classification Committee of the Society of American Bacteriologists for determining the identity of an organism the diastasic action presents the greatest difficulties. In all tests (seven are represented in the group number of the Society's classification card) of the activity of bacteria toward certain substances the aim is accuracy and ease of application. As at present carried out, the diastase test is far from satisfactory, not only because of the difficulty in obtaining potatoes of uniform quality, but also because of the difficulty arising when one tries to make a sharp distinction between "strong" and "feeble action." If the action is vigorous or if it is very feeble it is easily classed, but there are points between these extremes which produce doubt and the test then becomes a matter of mere judgment.

During the last two years in the Dairy Bacteriology Laboratory of the University of Illinois the use of potato slants for the determination of diastase production by bacteria has been replaced by a simple plate method as follows: A starch agar is made by adding 0.2 per cent of water-soluble starch to the regular plain agar. This starch agar can be sterilized in the autoclave along with other media. Some hydrolysis takes place, but not enough to interfere with the test. The agar is poured into Petri dishes and allowed to cool, when a stroke 2 inches long is made with a loopful of an agar slant growth of the organism to be tested. The plates are incubated for two days at 37°C. and five days at 20°C., after which they are flooded with a saturated solution of iodine in 50 per cent alcohol.



A clear area about the stroke averaging more than 2 mm. in width classifies the diastatic action as *strong,* while a width of 2 mm. or less marks it as *feeble* and the absence of a halo as *absent.* These are the three terms used on the classification card of the Society of American Bacteriologists. As the loopful of growth from an agar slant is the unit amount of inoculation which is used in all of the other tests which go to make up the group number of an organism, the starch agar test is consistent in this respect.

As used in this laboratory, some of the evident advantages of this method over the potato slant method are the decrease in time and labor in the preparation of the media, and the greater accuracy which the average worker can obtain.

The starch agar gives best results if used before it is two weeks old, as after that period of time the starch becomes changed and spots of reddish purple develop on the addition of iodine solution.

As to the comparison of this method with the potato test, it has been found that for the organisms of dairy products, approximately the same results are arrived at whether one test or the other is used.

A SIMPLE AND PRACTICAL MEDIUM FOR DIFFERENTIATING B. TYPHOSUS, B. PARATYPHOSUS A., B. PARATYPHOSUS B., B. ENTERITIDIS, AND B. COLI

KAN-ICHIRO MORISHIMA

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Before the war in Europe began paratyphoid fever was relatively rare, but owing partly to prophylactic inoculation against typhoid, it soon became more common among soldiers than the latter disease. With increased prevalence there is need of simple cultural tests for the recognition of the causative organisms. A number of such tests have been devised. Russell's double sugar medium, consisting of litmus agar with 1 per cent lactose and 0.1 per cent glucose, is widely used to receive the fishings of suspicious colonies. It differentiates the paratyphoids and *B. enteritidis* from *B. typhosus* and the *B. coli* group, but does not differentiate between *B. paratyphosus A*, *B. paratyphosus B.*, and *B. enteritidis*. To determine which of these latter organisms is present, further inoculation of the culture into litmus milk, lead acetate agar, or other special culture media, and agglutination tests must be resorted to.

It occurred to me that by pouring one agar medium into a test tube and allowing the agar to solidify, and then pouring a layer of a second differential agar medium upon this, a medium would be obtained which would furnish, in twenty four hours, more information from a stab fishing than do the media at present employed. Of a large number of such combinations, the following proved to be the most satisfactory.

Meat infusion agar is prepared in the usual way, cleared with egg-white, and titrated while hot to about -0.2 to -0.4 with phenolphthalein as an indicator. A 2 per cent solution of neu-

tral lead acetate in sterile distilled water is prepared and heated for one-half hour at 100°C. in a water bath. Five cubic centimeters of the solution is added to 100 cc. of the agar, melted and cooled to about 60°C. This lead acetate agar is transferred to small test tubes to a depth of about 1.5 cc. If the agar with the lead acetate is tubed while hot, a precipitate containing most of the lead settles to the bottom of the tube; this may be prevented by cooling the agar to 60° or 70°C. before tubing it.

A 1 per cent stock solution of china blue in distilled water is kept on hand. Four-tenths of a cubic centimeter of normal sodium hydrate are added to 10 cc. of the china blue solution, and the mixture is heated on the water bath for ten minutes at 100°C. The color changes from blue to brown during the heating. One and one-fifth cubic centimeters of this decolorized china blue solution is added to 100 cc. of nutrient agar of reaction, −0.2 to −0.4. One per cent lactose and 0.1 per cent glucose are added and the mixture is heated ten minutes in the water bath at 100°C. The medium is cooled to about 60°C, and is then ready to be added as a second layer to the small tubes containing the lead acetate agar. It forms a layer of about the same depth as the latter medium. The tubes are incubated overnight, and any contaminated ones are discarded.

The results of stab cultures in this medium after ten to eighteen hours' incubation may be summarized as follows:

CULTURE	GAS PRODUCTION	BOTTOM LAYER	TOP LAYER
B. typhosus	−	Black	Pale blue
B. paratyphosus A	+	No change	Pale blue
B. paratyphosus B	+	Black	Colorless
B. enteritidis	+	Black	Colorless
B. coli	++	Black, or no change	Deep blue

It is thus seen at a glance on the morning after the suspicious colony is fished, whether the organism is *B. paratyphosus A* or *B*, the typhoid bacillus, or *B. coli*. The medium does not differentiate between *B. paratyphosus B* and *B. enteritidis*. By in-

creasing the amount of the upper layer and slanting it, a streak culture can be made before stabbing, as in the case of the Russell medium, and material will thus be available for agglutination tests without further transplantation. The medium is readily prepared, and is inexpensive. It has been preserved for one month in the ice-box without deterioration; by adding two drops of sterile liquid paraffine to prevent evaporation, it can probably be kept indefinitely.

I next attempted to prepare a medium that would differentiate between *B. paratyphosus B* and *B. enteritidis*, as well as between these organisms and *B. paratyphosus A*, *B. typhosus*, and *B coli*. The following represents the best combination obtained for this purpose. Sugar-free agar is used for both the lead acetate and the china blue; 1 per cent lactose, 1 per cent inosite, and 0.1 per cent arabinose are added to the china blue agar. With this medium the lead acetate layer behaves toward the different organisms as in the preceding medium, and all of the organisms produce gas in it, except *B. typhosus*. The shade of blue produced in the upper layer after twenty-four hours' incubation may be indicated as follows:

B. typhosus	+	
B. paratyphosus A	+	
B. paratyphosus B. sub. 1	+ +	
B. paratyphosus B. sub. 2	–	or trace
B. enteritidis	–	or trace
B. coli	+ + +	

B. paratyphosus B has been divided into two groups by Weiss, according to whether the organism does or does not ferment inosite; most of the strains ferment inosite, and so fall into his sub 1 group. Therefore, the medium just described differentiates sharply between most paratyphoid B strains and *B. enteritidis* but not between paratyphoid B sub 2 and *B. enteritidis*. Fourteen of our paratyphoid B. strains belong to the sub 1 group, and only five to the sub 2 group. The medium differentiates quite sharply,—*B. enteritidis* and *B. paratyphosus* sub 2 remaining colorless or producing a mere trace of blue; *B. typhosus* and *B. paratyphosus B* showing pale blue; *B. paratyphosus B*

sub 1 becoming several shades deeper blue, and, finally, *B. coli* showing a dark blue.

The inosite used in these experiments was kindly furnished by Dr. W. G. Lyle. I am indebted to Dr. Oscar Teague for suggestions during the progress of this work.

A NEW APPARATUS FOR OBTAINING SIMULTANEOUS CULTURES OF ANY DESIRED AGE FOR COMPARATIVE STUDY

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From the Laboratories of Hygiene and Bacteriology, Western Reserve Medical School

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In the course of a series of investigations of the morphology of *B. diphtheriae*, it became necessary to study cultures of different ages on account of the well recognized fact that the morphology varies from hour to hour. It is of course simple enough to get eighteen-hour cultures, twenty-four-hour cultures and cultures five, six or seven hours old, but unless the investigator is living at or near the laboratory, there are many intervals which are very inconvenient. To avoid this difficulty, and to avoid also the multiplicity of inoculations necessary for any long series, an attempt was made to develop an apparatus which would give one a complete series of cultures of different ages with a single inoculation. This has been done successfully in the following manner.

Through the Waterbury Clock Company a small eight-day movement was obtained which had been so arranged that the hour hand revolved once in twenty-four hours instead of once in twelve hours, as do the twenty-four-hour clocks much used by foreign railroads. This was set horizontally and a table arranged to be carried by the twenty-four-hour wheel. The table was so fitted that vertical swab holders could be fastened at various points and would of course make a twenty-four-hour circle. On a vertically adjustable hanger an open inverted plate containing a solid medium was suspended so that the surface of the agar was parallel to and immediately above the swab-carrying table. When the sterile swab was inoculated with a given culture and by means of the adjustment brought into contact with the under

surface of the plate, and the clock started, a complete-circuit smear was made covering all time periods from nothing to twenty-four hours. By marking the bottom of the plate the exact age of any given point could readily be determined. The apparatus, as illustrated in the accompanying photographs, which are self-explanatory, has worked successfully, but requires certain precautions for its use. In the first place, unless the apparatus is contained in a practically saturated atmosphere, the plate will dry out. We therefore cover the machine with a bell jar set in a water seal at the base. In the earlier work a cotton swab inoculated with the culture was used, but a theoretical objection to this method was the possibility of growth in the swab, which might interfere with the accurate morphological time interpretations. Even so, it would be at least as accurate as the method of direct inoculations, which places organisms of all ages on the inoculated media. To obviate this possible criticism, sterile water with a suspension of the organisms was used, but this apparently took up sufficient nutriment from the culture medium to have a similar result, as indicated by a heavier growth in the later periods. We now use small flaps of sterile tinfoil about 0.5 cm. in width, and find that the capillary attraction of the surface of the medium gives a good continuous contact. By this means organisms are left at the margin of the sliding foil and develop under uniform conditions at all parts of the smear. Organisms, for instance, which have left the inoculating foil at a point half way round the plate are twelve hours old, and so on.

It is obvious that for satisfactory results the medium must be of a proper consistency, and that its surface and that of the swab-carrying table must be parallel. With these precautions it is easy to obtain an even smear with separate colonies along the track of the swab. At the end of the twenty-four hours one can remove the plate, and by the use of a series of sterile cover slips get impressions of the entire circle, which may readily be labeled so that any given hour of growth can be studied with considerable accuracy. If it is desired to study cultures twenty-four to forty-eight hours old, the plate can be removed from the

apparatus, covered, and returned to the incubator for twenty-four hours. The cultures first recorded as twelve hours will then be thirty-six hours old, and so on.

The apparatus appears to have a variety of other possible uses, inasmuch as it is easy to set up relay make-and-break connections, so that an incubator or a heating apparatus of any sort may be turned on and off as many times as desirable up to twenty-four hours instead of twelve, which is obviously a limit of insufficient extent. It is felt that applications of this character may be of a good deal of value in the study of conditions involving time comparisons, and it is hoped that others may be able to find the scheme of assistance in their work. The original apparatus was constructed by the writer out of scrap materials at a total expense, including the clock ($5.50) and exclusive of time, of about $6. The improved apparatus was cast in aluminum and built up by the laboratory technical assistant at an expense, exclusive of labor and inclusive of the clock, of about $11. Details of model and construction will gladly be furnished to those interested.

DESCRIPTION OF PLATES 1 AND 2

The numbers on both plates refer to the same parts.

1, Main frame containing clock and with gutter for water seal. The number in the detailed print lies in the gutter.

2, Cover over clock with openings for shafts and regulator.

3, Revolving table turning once in twenty-four hours.

4, Clamp for Petri plate, with vertical and horizontal adjustments to fit any plate from 2 to 4 inches in diameter.

5, Vertical shaft with rack to fit pinion adjustment under No. 4.

NOTE.—This rack and pinion was taken from an old microscope. The openings in No. 2 should be covered during use by an oiled felt washer to keep the steel parts of the clock from rusting in the saturated atmosphere.

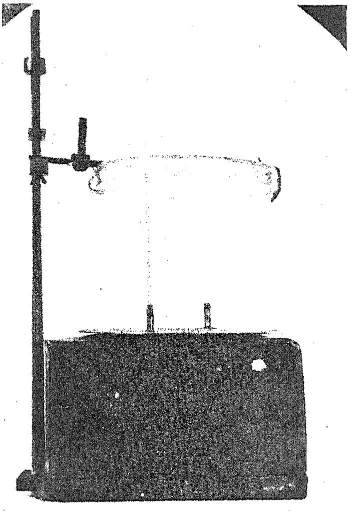

FIG. 1. ORIGINAL MODEL

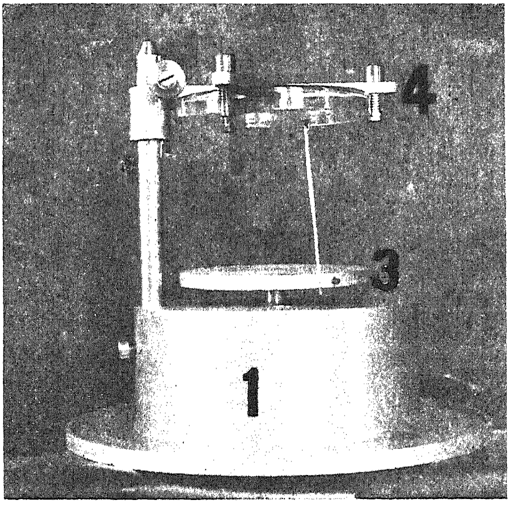

FIG. 2. PRESENT MODEL

(Perkins: Obtaining Simultaneous Cultures)

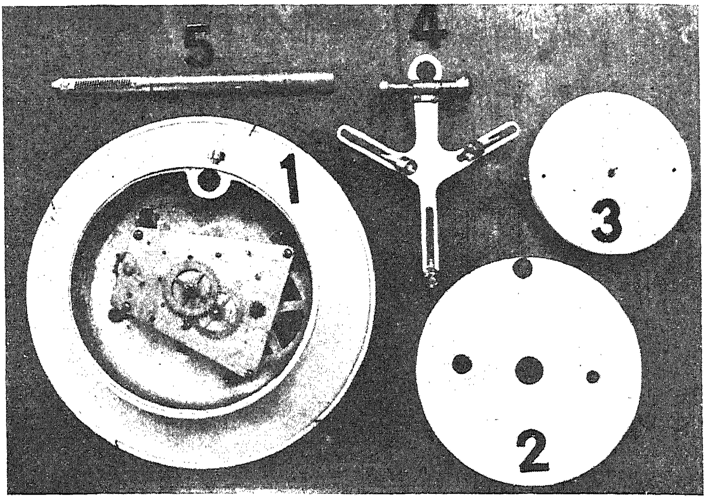

FIG. 3. DETAILED PRESENT MODEL

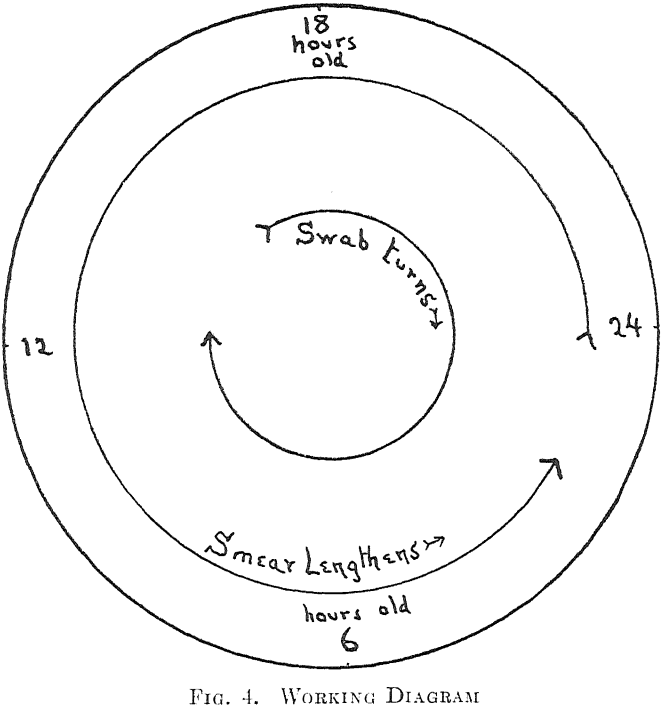

FIG. 4. WORKING DIAGRAM

(Perkins: Obtaining Simultaneous Cultures)

STUDIES IN THE NOMENCLATURE AND CLASSIFICATION OF THE BACTERIA

V. SUBGROUPS AND GENERA OF THE BACTERIACEAE

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From the Bacteriological Laboratories of Iowa State College, Ames, Iowa

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Family II. Bacteriaceae Cohn, 1872a, p. 237

Synonyms:

Bacterina Perty, 1852, p. 159
Microbacteria Cohn, 1872, p. 167
Desmobacteria Cohn, 1872, p. 173
Bacteriaceen Zopf, 1883, p. 45
Baculogenae Trevisan, 1885, p. 92
Bacteriacei Schroeter, 1886, p. 155
Bakteriaceen Hueppe, 1886, p. 142
Arthrobakteriaceen Hueppe, 1886, p. 149
Bacteriacees Cornil and Babes, 1890, p. 151
Bacillaceae Fischer, 1895, p. 139
Bacillacei Fischer, 1895, p. 139.

Cells rod-shaped, straight, or at least not spherical or spiral. Never containing sulphur granules, nor with bacterio-purpurin, many species pigmented. May or may not be motile by polar or diffuse flagella. Cells may be single or in chains. Endospores produced in some genera, not in others. A pseudoplasmodium never developed. Growth energy not secured by the oxidation of ammonia or nitrites.

The following names have been used for subdivisions of *Bacteriaceae* as here defined:

Bacterieae Trevisan, 1879, p. 136
Eubacterieae Trevisan, 1879, p. 136

Bacilleae Trevisan, 1889, p. 12.
Eubacilleae Trevisan, 1889, p. 939
Klebsielleae Trevisan, 1889, p. 1028
Kurthieae Trevisan, 1889, p. 929
Pasteurieae Trevisan, 1889, p. 12
Bactrillei Fischer, 1895, p. 139
Bactrinei Fischer, 1895, p. 139
Bacillei Fischer, 1895, p. 139
Mycobacteriaceae Chester, 1897, p. 63
Clostridieae Fischer, 1903, p. 60
Plectridieae Fischer, 1903, p. 61
Acidobacteriaceae Jensen, 1909a, p. 344
Alkalibacteriaceae Jensen, 1909a, p. 343
Butyribacteriaceae Jensen, 1909a, p. 343
Luminobacteriaceae Jensen, 1909a, p. 303
Putribacteriaceae Jensen, 1909a, p. 343
Oxydobacteriaceae Jensen, 1909a, p. 343

De Toni and Trevisan (1889, p. 939) separated the various tribes and subtribes of their subfamily, *Baculogenae* in the following manner:

Key to Tribes and Subtribes of Baculogenae. DeToni and Trevisan

A. Bacilli and cocci naked, never with a capsule.............Tribe I. *Bacilleae*
    I. Producing endospores.
        1. Division longitudinal................Subtribe 1. *Pasteurieae*
        2. Division transverse.
            a. Rods united into a reticulate coenobium
                Subtribe 2. *Thiodictyeae*
            b. Rods not united into a reticulate coenobium.
                (1) Rods never spiral..........Subtribe 3. *Eubacilleae*
                (2) Rods spirally bent..........Subtribe 4. *Spirilleae*
    II. Producing arthrospores....................Subtribe 5. *Pacinieae*
B. Bacilli and cocci surrounded by a special membranous or gelatinous capsule..Tribe II. *Klebsielleae*
    I. Rods straight or curved, never true spirals.
        Subtribe 1. *Euklebsielleae*
    II. Rods spirally twisted..................Subtribe 2. *Myconostoceae*

Of these names *Pasteurieae* may be discarded because *Pasteuria* is probably a protozoan. *Thiodictyeae* includes forms

belonging to *Thiobacteria; Spirilleae, Pacineae,* and *Myconostoceae* are based upon genera now placed with the *Spirillaceae.*

Fischer (1895, p. 139) subdivided his family *Bacillacei* as follows:

Key to subfamilies of Bacillacei. Fischer

A. Non motile. Non flagellate..I. *Bacillei*
B. Motile. With flagella
  I. Polar flagella only.
    a. A single polar flagellum........................II. *Bactriniei*
    b. A tuft of polar flagella........................III. *Bactrillei*
  II. With diffuse flagella................................IV. *Bactridiei*

Chester (1897, p. 63) grouped certain genera to constitute the family *Mycobacteriaceae.* The most important differential characters are the formation of clavate-cuneate cells, lack of endospores, and true dichotomous branching.

Later Fischer (1903, p. 60) separated his family *Bacillaceae* into three subfamilies as follows:

Key to subfamilies of Bacillaceae. Fischer

A. Spore-bearing rods unchanged in shape, cylindrical. Includes all non-sporulating forms....................................Subfamily I. *Bacilleae*
B. Spore-bearing rods modified in shape.
  I. Spore-bearing rods spindle shaped......Subfamily II. *Clostridieae*
  II. Spore-bearing rods drumstick shaped..Subfamily III. *Plectridieae*

The families proposed by Jensen (1909a, p. 344) which would be included under our definition of *Bacteriaceae* may be separated by the following key:

Key to the families of Bacteriaceae. Fischer

A. Without spores, motile or non-motile; when the former, with polar flagella, typically water forms, securing energy almost exclusively by oxidative processes.
  I. Obligate aërobes, oxidizing carbon, hydrogen or nitrogen compounds without production of noteworthy amounts of unoxidized split products.........................I. *Oxydobacteriaceae*
  II. Luminous and fluorescent bacteria.........II. *Luminobacteriaceae*
B. With or without spores, either peritrichous or non-motile, not typically water forms, do not secure energy exclusively as "A."
  I. Typically not obligate anaërobic or micro-aërophilic.
    a. Usually producing acids from carbohydrates.
      I. *Acidobacteriaceae*
    b. Usually bringing about an alkaline reaction by the development of ammonia...................II. *Alkalibacteriaceae*

II. Typically anaërobic or micro-aërophilic.
- a. Characteristically producing butyric acid from carbohydrates.............................I. *Butyribacteriaceae*
- b. Characteristically bringing about putrefactive changes in proteins.............................II. *Putribacteriaceae*

An examination of the genera which have been proposed by various authors would seem to indicate that an important differential character is that of endospore production. Separation upon this basis throws all the spore-bearing rods together, a grouping which appears to be wholly natural and to represent true relationships. Of the rod-shaped organisms which do not produce spores it seems that the acid-fast bacteria related to the tubercle bacillus likewise represent a very distinct group.

For the three tribes of the *Bacteriaceae* the names *Bacilleae* *Bacterieae,* and *Mycobacterieae* may be used. The principal differential characters may be summarized in the following key:

Key to the Tribes of the Bacteriaceae

A. Cells not acid-fast.
- I. Typically producing endospores under favorable conditions. Tribe I. *Bacilleae*
- II. Not producing endospores....................Tribe II. *Bacterieae*

B. Cells acid-fast, frequently showing some tendency to branching Tribe III. *Mycobacterieae*

Tribe I. **Bacilleae** Trevisan 1889, p. 12 in part

Synonyms:

Eubacilleae Trevisan, 1889, p. 939 in part
Bactrillei Fischer, 1895, p. 139 in part
Bacillei Fischer, 1895, p. 139 in part
Bactrinei Fischer, 1895, p. 139 in part
Bacilleae Fischer, 1903, p. 60 in part
Clostrideae Fischer, 1903, p. 60 in part
Plectrideae Fischer, 1903, p. 61 in part
Alkalibacteriaceae Jensen, 1909a, p. 343 in part
Butyribacteriaceae Jensen, 1909a, p. 343 in part
Putribacteriaceae Jensen, 1909a, p. 343 in part

Cells rodshaped, never spiral or strictly filamentous, single or in

chains, usually motile by means of peritrichous flagella, producing endospores under suitable conditions of growth, usually Gram-positive.

The following pseudogeneric names of organisms within this tribe have been employed, but never have been used with a species or in a strict generic sense, and are not valid as genera.

Amylobacter Trecul, 1865, p. 432
Urobacter Trecul, 1865, p. 432
Urocephalum Trecul, 1865, p. 432
Megabacterium Billroth, 1874, p. 16
Mesobacterien Billroth, 1874, p. 16
Petalobacteria Billroth, 1874, p. 16
Streptobacteria Billroth, 1874, p. 18
Microbacterien Billroth, 1874, p. 18
Microbacteria Billroth, 1874, p. 18
Aethylbacillus Fitz, 1878, p. 48
Polybacteria Van Tieghem, 1884
Streptobacterium Billet, 1890, p. 23
Streptobacillus Preisz? Migula, 1900, p. 374
Bacterius Kendall, 1902, p. 484
Bactrillus Kendall, 1902, p. 484
Bactrinius Kendall, 1902, p. 484
Microbacterium (Billroth) Smith, 1905, p. 174
Plennobakterium Gonnermann, 1907, p. 887

The following generic names have been used, but the species are not identifiable with any degree of certainty:

Bactrella Morren, 1830, p. 1
Sporonema Perty, 1852, p. 160

Subgeneric names:

Streptobacter Schroeter, 1886, p. 158
Eu-Cornilia Trevisan, 1889, p. 998
Pleurospora Trevisan, 1889, p. 1002

The following generic name is invalid because earlier used as the generic name of a distinct group of plants.

Bactridium Fischer, 1895, p. 139
not *Bactridium* Kunze
not *Bactridium* Salisb.

Generic names which are not invalid for any of the preceding reasons are the following:

Serratia Bizio, 1823, p. 288
Bacterium Ehrenberg, 1828
Bacteridium Davaine, 1868, p. 21
Bacillus Cohn, 1872b, p. 174
Urobacillus Miquel, 1879, p. 517
Clostridium Prazmowski, 1880, p. 23
Pollendera Trevisan, 1884, p. 943
Zopfiella Trevisan, 1885, p. 93
Cornilia Trevisan, 1889, p. 21
Eubacillus Dangeard, 1891, p. 151
Granulobacter Beijerinck, 1894, p. 3
Bactrillum Fischer, 1895, p. 139
Bactrinium Fischer, 1895, p. 139
Clostrillum Fischer, 1895, p. 139
Clostrinium Fischer, 1895, p. 139
Diplectridium Fischer, 1895, p. 140
Paracloster Fischer, 1895, p. 141
Paraplectrum Fischer, 1895, p. 141
Plectridium Fischer, 1895, p. 147
Astasia Meyer, 1898, p. 49
Fenobacter Beijerinck, 1900, p. 200
Aplanobacter E. F. Smith, 1905, p. 171
Semiclostridium Maassen, 1905, p. 5
Myxobacillus Gonnermann, 1907, p. 877
Botulobacillus Jensen, 1909a, p. 343
Butyribacillus Jensen, 1909a, p. 343
Cellulobacillus Jensen, 1909a, p. 343
Putyribacillus Jensen, 1909a, p. 343
Pectobacillus Jensen, 1909a, p. 343
Metabacterium Chatton and Perard, 1913, p. 1232

The genus *Eubacillus* Dangeard is believed by Migula (1897,

p. 94) to include organisms which are not true bacteria. The type species, *Eubacillus multisporus*, has chlorophyll, is filamentous, and produces many endospores in a filament. Until this organism can again be identified and studied more closely its position is doubtful, and it should perhaps be excluded from present consideration.

The genera of the tribe *Bacilleae* may be differentiated by the following key:

Key to the genera of Bacilleae

A. Usually a single endospore formed within each cell.
   - I. Aërobic, usually Gram-positive, as a rule liquefying gelatin, spores usually not distorting rods when formed.......Genus 1. *Bacillus*
   - II. Anaërobic or microaërophilic usually.
     - a. Spores produced at extreme tip of cells, forming typical drumsticks............................Genus 2. *Plectridium*
     - b. Spores not produced at extreme tip of cells, at least not forming drumsticks. Cells usually somewhat swollen when spores are formed.....................Genus 3. *Clostridium*

B. Usually a number of spores develop within a swollen cell.
Genus 4. *Metabacterium*

Genus 1. **Bacillus** Cohn, 1872b, p. 174

Synonyms:

Bactrella? Morren, 1830, p. 354
Metallacter? Perty, 1852, p. 180
Bacteridium Davaine, 1868, p. 21
Urobacillus Miquel, 1879, p. 517
Pollendera Trevisan, 1884, p. 943
Dispora? Kern, 1882, p. 135
Zopfiella Trevisan, 1885, p. 93
Cornilia Trevisan, 1889, p. 21 in part
Bacterium Migula, 1894, p. 237 in part
  not *Bacterium* Ehrenberg, 1828
  not *Bacterium* Cohn, 1872b, p. 167
Bactrinium Fischer, 1895, p. 139
Bactrillum Fischer, 1895, p. 139
Endobacterium Lehmann and Neumann, 1896, p. 103
Astasia Meyer, 1898, p. 49
Saccharobacter? Beijerinck, 1900, p. 200

Fenobacter Beijerinck, 1900, p. 200
Aplanobacter E. F. Smith, 1905, p. 171
Semiclostridium Maassen, 1905, p. 5
Myxobacillus Gonnermann, 1907, p. 877
Plennobacterium Gonnermann, 1907, p. 887
Serratia Vuillemin, 1913, p. 521
not *Serratia* Bizio 1823

Cells rod-shaped, straight or at least never spiral, motile by diffuse flagella or non-motile. Endospores produced under favorable conditions, not usually distorting the cell, usually Gram-positive. Growth usually good on laboratory media; commonly liquefying gelatin. Aërobic or facultative.

There has been much diversity in the literature as to the use of the generic name *Bacillus*. It was first used by Cohn (1872, p. 174) to include three species of rod-shaped organisms, *Bacillus subtilis*, *B. ulna*, and *B. anthracis*. He characterized the genus as including those rod-shaped organisms that grow in filaments. Later he discussed at length spore production in *B. subtilis*, and gave the first accurate description of endospores. Various authors accepting either *B. subtilis* or *B. anthracis* as the type of the genus have emphasized different characters so that there are current in literature at least six conceptions of the genus. These are as follows:

1. Bacillus. Rod-shaped organisms growing in filaments or in chains. Spore production, flagella distribution and motility not emphasized or regarded as secondary. Among the authors who have made use of this definition are Cohn (1872 and 1875), Magnin (1878), Winter (1879), Luerssen (1879), Van Tieghem (1884), Grove (1884), Flügge (1886), and Schroeter (1886).

2. Bacillus. Rod-shaped organisms producing endospores. (Some authors recognize other spore-bearing genera in addition to *Bacillus*.) The following have used this definition; De Bary (1884 and 1887), Zopf (1885), Hueppe (1885), Cornil and Babes (1885 and 1890), De Toni and Trevisan (1889), Ludwig (1892), Freudenreich (1894), Lehmann and Neumann (1896), Chester (1897), Flügge (1908), Jensen (1909), Heim (1911), Löhnis (1913).

3. Bacillus. Rods motile by means of peritrichous flagella, spores may or may not be produced. The following authors have recognized this definition; Migula (1894, 1895, 1897, 1900, 1904), Chester (1901), Kendall (1901), A. J. Smith (1902), E. F. Smith (1905), Ellis (1909), Frost (1911), and Schneider (1912).

4. Bacillus. Rods, non-motile, producing endospores. Fischer (1895), Lotsy (1907).

5. Bacillus. Any rod-shaped organism. Baumgarten (1890), Sternberg (1892), Mace (1897), Hewlett (1898), and Matzuschita (1902).

6. Bacillus. Any motile rod. Conn (1909).

Vuillemin (1913) has claimed that the name Bacillus has been so vulgarized by the bacteriologist because of the number of definitions that it should be abandoned as a generic designation. It is, of course, true that bacillus is used as a common designation of all rod-shaped bacteria, but this should not invalidate the use of this term as a generic name any more than the use of *Chrysanthemum* or *Aster* as genera by botanists is interfered with by the common names chrysanthemum and aster.

In some form or with some definition the genus *Bacillus* should be retained. The type practically always accepted is *B. subtilis.* The definition of Fischer should therefore be abandoned as including only non-motile forms. He would exclude from the genus its first described species. The original description of Cohn is scarcely sufficient, for much stress was laid upon cell grouping and length of cell and not upon other characters. The use of Migula's diagnosis, including in the genus all rods with peritrichous flagella, is the cause of great confusion. It brings into the genus such discordant types as the hay bacillus and the typhoid bacillus while it excludes the anthrax bacillus so closely related to the hay bacillus. Migula's definition should be abandoned as not based upon natural affinities. The definitions which would include all rods in the genus *Bacillus* have the merit of simplicity. When, however, organisms so diverse in characteristics as the tubercle bacillus, the typhoid bacillus, the tetanus bacillus, and the anthrax bacillus are all included in one genus the simplicity is more apparent than real. The existence

of such diverse forms has led most recent authors to divide bacteria into well marked groups. It is the opinion of the author that the larger of these groups should be recognized as genera. The term *Bacillus* should therefore be restricted, and it would seem that it should be defined more nearly in the terms of De Bary, Zopf, Hueppe, etc., who emphasized the importance of spore production as a diagnostic character.

The objection may be raised that a definition of *Bacillus* as a genus made up of endosporous rods would exclude forms which have lost the power of spore formation but are in other respects closely related. It is evidently impracticable to base generic diagnoses upon a single character. Even though an organism be a variant in one or even more characters, the other resemblances would be sufficient to include the organism in question in the correct genus. Illustrations of this fact may be taken from higher plants. The Lombardy poplar is always classified in the genus *Populus*. It never produces fruit, and it persists solely as the result of vegetative reproduction; yet the genus *Populus* is based in part upon certain fruit characters. The other characters are so evidently poplar-like, however, that we do not question the correctness of the assignment of this species to the genus *Populus*.

The genus *Bacillus* is a relatively large one, and shows some degree of differentiation among the species. It may to advantage be divided into subgenera.

The following key differentiates the subgenera on the basis of their most striking characteristics:

Key to the subgenera of Bacillus

A. Spore not barrel-shaped in longitudinal section and not star-shaped in cross section.
- I. Motile by means of peritrichous flagella..Subgenus 1. *Eu-Bacillus*
- II. Non-motile..............................Subgenus 2. *Bacteridium*

B. Spore barrel-shaped in longitudinal section, longitudinal striations evident. Subgenus 3. *Astasia*

Subgenus 1. Eu-Bacillus

Synonyms:

Urobacillus Miquel, 1879, p. 517
Zopfiella Trevisan, 1885, p. 93

Bactrinium Fischer, 1895, p. 139
Bactrillum Fischer, 1895, p. 139
Fenobacter Beijerinck, 1900, p. 200
Myxobacillus Gonnermann, 1907, p. 877
Serratia Vuillemin, 1913, p. 521
not *Serratia* Bizio, 1823

Motile, usually by means of peritrichous flagella. Spores not barrel-shaped, without longitudinal ridges. Other characters those of the genus.

The type species of the subgenus is *Bacillus subtilis* Cohn.

Subgenus 2. **Bacteridium** Davaine, 1868, p. 21

Synonyms:
Pollendera Trevisan, 1884
Bacterium Migula, 1894, p. 237
not *Bacterium* Ehrenberg, 1828
not *Bacterium* Cohn, 1872b, p. 167
Bacillus Fischer, 1895, p. 139
not *Bacillus* Cohn, 1872, p. 174
Aplanobacter E. F. Smith, 1905, p. 171

Non-motile, without flagella. Spores not barrel-shaped, without longitudinal ridges. Other characters those of the genus.

The type species is *Bacillus* (*Bacteridium*) *anthracis* Cohn.

The name *Bacteridium*, according to E. F. Smith, is invalid because of the previous existence of the genus *Bactridium*, Kunze, 1817. Whether or not this invalidates *Bacteridium* Davaine depends upon the interpretation of Article 57 and Recommendation XXXI of the botanical code. The former states that when the difference between two names, especially two generic names lies in the termination these names are to be regarded as distinct even though differing by one letter only. The latter reads:

Many names differ by a single letter without risk of confusion. In cases where a close approach to identity is a source of error (ex. *Astrostemma* and *Asterostemma* in one and the same family, *Asclepiadaceae*)

only one, the older, of the names should be kept in accordance with Article 51, 4°."

This last article reads:

Every one should refuse to admit a name in the following cases:
4. When the group it designates embraces elements altogether incoherent, or when it becomes a permanent source of confusion or error.

It would seem that there is ample justification in the code for the recognition of *Bacteridium* Davaine.

Subgenus 3. **Astasia** Meyer, 1898, p. 49

Motile rods, spores ovoid with longitudinal striae, star-shaped in cross section.

The type species is *Bacillus* (*Astasia*) *asterospora* Meyer.

Genus 2. **Plectridium** Fischer, 1895, p. 147

Synonyms:
Paraplectrum Fischer, 1895, p. 139
Plectrinium Fischer, 1895, p. 139
Plectrillum Fischer, 1895, p. 139
Diplectridium Fischer, 1895, p. 140
Putribacillus Jensen, 1909a, p. 343 in part
Pectobacillus Jensen, 1909a, p. 343 in part

Cells rod-shaped, straight or at least never spiral, motile by diffuse flagella or non-motile. Endospores produced under favorable conditions, causing an enlargement of one tip of the cell, giving rise to a drumstick appearance. Usually Gram-positive. Anaërobic or microaërophilic.

The type species is *Plectridium tetani* (Nicolaier) Fischer.

Genus 3. **Clostridium** Prazmowski, 1880, p. 23

Synonyms:
Amylobacter Trecul, 1865, p. 435
Cornilia Trevisan, 1889, in part

Granulobacter Beijerinck, 1893, p. 7
Clostrillum Fischer, 1895, p. 139
Clostrinium Fischer, 1895, p. 139
Paracloster Fischer, 1895, p. 140
Semiclostridium Maassen, 1905, p. 5
Botulobacillus Jensen, 1909a, p. 343
Butyribacillus Jensen, 1909a, p. 342
Cellulobacillus Jensen, 1909a, p. 343
Putribacillus Jensen, 1909a, p. 343 in part

Cells rod-shaped, straight or at least never spiral. Frequently showing granules. Endospores usually produced in cells showing some enlargement; usually the cells become spindle-shaped. Anaërobic or microaërophilic. Usually Gram-positive.

The type species is *Clostridium butyricum* Prazmowski.

Genus 4. **Metabacterium** Chatton and Perard, 1913, p. 1232

Rod-shaped, known only in the sporulating state, from the caecum of a guinea pig. Sporogenous cell becomes ellipsoidal with one to eight endospores within a single cell.

The type species is *Metabacterium polyspora* Chatton and Perard.

The organism has not been cultivated. The diagnosis makes it evident that the organism is scarcely sufficiently known to make accurate diagnosis possible. It has been urged by Vuillemin (1913) as a *genus conservandum.*

Tribe II. **Bacterieae** Trevisan, 1879, p. 136 emended

Synonyms:
Klebsielleae Trevisan, 1889, p. 1028
Acidobacteriaceae Jensen, 1909a, p. 303 in part
Luminibacteriaceae Jensen, 1909a, p. 344

Cells rod-shaped, never spiral nor strictly filamentous; single or in chains, motile or non-motile, never producing endospores, either Gram-positive or -negative.

The following pseudogeneric names or words not used strictly in a generic sense have been applied to organisms of this subfamily.

Mycothrix Itzigsohn, 1867
Microsporon Klebs, 1871
Ascobacteria Billroth, 1874, p.
Coccobacteria Billroth, 1874, p. 1
Diplobacteria Billroth, 1874, p. 16
Gliabacteria Billroth, 1874, p. 5
Megabacteria Billroth, 1874, p. 16
Mesobacteria Billroth, 1874, p. 16
Microbacteria Billroth, 1874, p. 16
Monobacteria Billroth, 1874, p. 16
Petalobacteria Billroth, 1874, p. 16
Polybacteria Van Tieghem, 1884, p. 1114
Punctula Van Tieghem, 1884, p. 1114
Diplobacterium Hasse, 1887, p. 347
Gliscrobacterium Malerba and Sanna Salaris, 1888, p. 486
Photobacter Beijerinck

The following names have been designated as genera, but without species.

Arthrobacterium De Bary, 1884
Arthrobacter Fischer, 1895, p. 141
Arthrobactridium Fischer, 1895, p. 140
Arthrobactrillum Fischer, 1895, p. 139
Arthrobactrinium Fischer, 1895, p. 139
Thermobacterium Fuhrmann, 1906, p. 8

The following generic names are old algal or protozoan genera to which bacteria were occasionally ascribed.

Monas Mueller, 1773, p. 3
Gloeotila Kuetzing, 1843, p. 245
Mycothamnion Kuetzing, 1843, p. 126

The following are aberrant spellings of generic names.

Kokkobacillus Biedert, 1885, p. 439
Arthrobakterium Hueppe, 1886, p. 145
Glischrobacterium Rothmann, 1904, p. 491
Bakterium of many German authors

The following are subgenera:

Eu-Klebsiella Trevisan, 1889, p. 1028
Eu-Mantegazzaea Trevisan, 1889, p. 942
Eu-Pacinia Trevisan, 1889, p. 23
Eupseudomonas Migula, 1895, p. 29

The following names are not invalid for any of the preceding reasons.

Mycoderma Persoon, 1822, p. 96
Serratia Bizio, 1823
Zaogalactina Sette, 1824, p. 51
Bacterium Ehrenberg, 1828, p. 8
Ulvina Kuetzing, 1837, p. 26
Umbina Naegeli, 1849
Zoogloea Cohn, 1854, p. 123
Schinzia Frank, 1879, p. 376
  not *Schinzia* Dennstatt, 1818
  not *Schinzia* Naegeli, 1842, p. 279
Mantegazzaea Trevisan, 1879, p. 146
Cromobacterium Bergonzini, 1881, p. 153
Tyrothrix Duclaux, 1879, p. 79
Actinobacter Duclaux, 1882, p. 110
Dispora Kern, 1882, p. 135
Bacteriopsis Trevisan, 1885, p. 103
Coccobacillus Leube, 1885
Klebsiella Trevisan, 1885, p. 94
Kurthia Trevisan, 1885, p. 92
Octopsis Trevisan, 1885, p. 102
Proteus Hauser, 1885, p. 100
Helicobacterium Miller, 1886, p. 117
Phytomyxa Schroeter, 1886, p. 134
Diplobacillus Weichselbaum, 1887, p. 212
Pasteurella Trevisan, 1887, p. 94
Zygobacterium Maggio, 1887, p. 318
Cladochytrium Vuillemin 1888, p. 121
Coccobacterium Rivolta, 1888.
Diccoccia Trevisan 1889, p. 26

Photobacterium Beijerinck 1889, p. 401
Pneumobacillus Arloing, 1889, p. 428
Rhizobium Frank, 1889, p. 338
Winogradskya Trevisan, 1889, p. 12
Ascobacterium Babes, 1890, p. 155
Nevskia Famintzin, 1891, p. 481
Pseudomonas Migula, 1894, p. 237
Halibacterium Fischer, 1894, p. 22
Rhizobacterium Kirchner 1895, p. 213
Oenobacillus Forti, 1896, fasc. 1
Corynebacterium Lehmann and Neumann, 1896, p. 350
Astrobacter Jennings, 1896, p. 312
Aërobacter Beijerinck, 1900, p. 198
Pseudorhizobium Hartleb, 1900, p. 887
Azotobacter Beijerinck, 1901, p. 561
Corynethrix Bongert 1901, p. 449
Lactobacillus Beijerinck 1901b, p. 214
Lactobacter Beijerinck 1901b, p. 200
Brachybacterium Troili-Peterson, 1903, p. 138
Thiobacillus Beijerinck, 1904, p. 598
Acetobacter Fuhrmann, 1905, p. 8
Erythrobacillus Fortineau, 1905, p. 104
Pyobacillus Koppanyi, 1907, p. 429
Acetimonas Jensen, 1909a, p. 312
Carboxydomonas Jensen, 1909a, p. 311
Caseobacterium Jensen, 1909a, p. 311
Corynemonas Jensen, 1909a, p. 344
Denitrobacterium Jensen, 1909a, p. 314
Denitromonas Jensen, 1909a, p. 314
Erysipelothrix Rosenbach, 1909, p. 367
Liquidobacterium Jensen, 1909a, p. 337
Liquidomonas Jensen, 1909a, p. 332
Methanomonas Jensen, 1909a, p. 311
Propionibacterium Jensen, 1909a, p. 337
Rhizomonas Jensen, 1909a, p. 334
Thermobacillus Jensen, 1909a, p. 339
Asterococcus Borrel, etc., 1910, p. 179

Fusiformis Hoelling, 1910, p. 240
Thermobacterium Lindner
Microbacillus Sabouraud
Photobacter Beijerinck
Ascobacillus Unna and Tommasoli, p. 60

Among this list of generic names the following are based upon species insufficiently described, and are disregarded here: *Actinobacter*, *Astrobacter*, *Coccobacterium*, *Helicobacterium*, *Winogradskya*, *Zoogloea*, *Zygobacterium*.

The following have been termed physiological genera and have usually been regarded as invalid: *Halibacterium*, *Photobacter*, *Photobacterium*, *Lactobacter*.

The following generic names have not been included for other reasons: *Ascobacillus*, *Ascobacterium*, *Carboxydomonas*, *Oenobacillus*, *Photobacterium*, *Methanomonas*, *Microbacillus*.

The following key gives the principal differential characters of the subtribes which are proposed:

Key to the subtribes of the Bacterieae

A. Cells usually fusiform............................Subtribe I. *Fusiforminae*
B. Cells not fusiform.
    I. Requiring serum or hemoglobin for development. Obligate parasites. Gram-negative. Non-motile..Subtribe II. *Hemophilinae*
    II. Not requiring serum, or at least hemoglobin for development. Gram-negative or positive. Motile or non-motile.
        a. Obligate aërobes, securing growth energy by oxidation of carbonaceous compounds, as carbohydrates, alcohols, etc.............................Subtribe III. *Rhizobiinae*
        b. Not obligate aërobes, not securing growth energy by oxidation of carbonaceous compounds, Subtribe IV. *Bacteriinae*.

Subtribe I. **Fusiforminae** Subtrib. nov.

This tribe has a single genus, *Fusiformis* Hoelling.

Genus I. **Fusiformis** Hoelling 1910, p. 240

Synonym:
Mantegazzaea Vuillemin 1913, p. 521
not *Mantegazzaea* Trevisan 1879, p. 137

Obligate parasites. Cells usually elongate and fusiform. Gram-negative? Anaërobic. Non-motile. No spores. In some respects approaching the spirochetes in morphology.

The type species is *Fusiformis termitidis* Hoelling (possibly *F. dentium*).

Vuillemin (1913, p. 521) has proposed that the name *Mantegazzaea* Trevisan be retained as one of the "formogenera conservanda," with the fusiform bacillus of the medical writers as the type species. The name as used by Trevisan was used principally for certain of the sulphur bacteria, and not for forms related to this type.

Subtribe II. **Hemophilinae** Subtrib. nov.

Strict parasites, requiring hemoglobin or at least serum for their growth in media. Gram-negative. Non-motile. Cells may be pleomorphic. Usually very small. No spores.

The two genera may be differentiated by the following key:

Key to the genera of Hemophilinae

A. Requiring serum for growth. Cells almost ultra-microscopic. Stain best by Giemsa. Involution forms characteristic.........Genus 1. *Asterococcus*

B. Requiring hemoglobin for growth. Stain readily with ordinary aniline dyes. Involution forms infrequent...................Genus 2. *Hemophilus*

Genus I. **Asterococcus** Borrel, *et al.*, 1910, p. 179

Pleomorphic cells, appearing at different stages of development as isolated cocci or as chains of cocci, as rods and as filaments variously branched and swollen. Very minute, almost ultra-microscopic. Non-motile. No spores. Stains with difficulty, best with Giemsa stain. Growth in cultures only in presence of serum or of hemoglobin.

The type species is *Asterococcus mycoides* Borrel, *et al.*, of bovine pleuropneumonia.

Genus 2. **Hemophilus.** Committee

Rod-shaped cells, minute, non-motile, without spores, strict parasites, growing best or only in presence of hemoglobin. Gram-negative, stain readily with aniline dyes.

The type species is *Hemophilus influenzae*, (——) comb. nov. the cause of influenza.

Subtribe III. **Rhizobiinae** Subtrib. nov.

Rod-shaped organisms, securing their growth energy by the oxidation of carbonaceous compounds, as carbohydrates, alcohol, etc. Do not require serum, etc. Not parasitic in animals.

The following key gives the principal differential characters of the genera:

Key to the genera of Rhizobiinae

A. Not fixing atmospheric nitrogen; securing growth energy usually by the oxidation of ethyl alcohol to acetic acid...............Genus 1. *Mycoderma*

B. Capable of fixing appreciable amounts of atmospheric nitrogen. Grow well on nitrogen-free media.
   - a. Small motile rods, with abundant involution forms, frequently living in root nodules of the higher plants (legumes)...Genus 2. *Rhizobium*
   - b. Not symbiotic. Cells larger, plump, almost spherical in some cases. Genus 3. *Azotobacter*

Genus I. **Mycoderma** Persoon, 1822, p. 96 emended

Synonyms:

Ulvina Kuetzing, 1837, p. 26
Umbina Naegeli, 1849
Bacteriopsis? Trevisan, 1885, p. 103
Acetobacter Fuhrmann, 1905, p. 8
Acetimonas Jensen, 1909a, p. 312

Cells rod-shaped, frequently in chains, non-motile usually, without spores. Obligate aërobes, growing usually as a film on the surface of alcoholic solutions, transforming the alcohol to acetic acid. Involution forms often developed and quite characteristic.

The type species is *Mycoderma aceti* Thompson?

There is some doubt as to the appropriateness of *Mycoderma* as the name of this genus. It is possible that it should be reserved for the yeasts.

Genus 2. **Rhizobium** Frank, 1889, p. 338

Synonyms:

Cladochytrium Vuillemin, 1888, p. 121
Phytomyxa Schroeter, 1886, p. 134
Schinzia Denstatt, 1818, in part
Rhizobacterium Kirchner, 1895, p. 213
Pseudorhizobium Hartleb, 1900, p. 887
Rhizomonas Jensen, 1909a, p. 334

Rod-shaped cells, motile when young by means of polar flagella, obligate aërobes, fixing atmospheric nitrogen when grown in a nitrogen-free medium containing suitable carbohydrates, involution forms abundant and characteristic, usually growing in the nodules of the roots of leguminous plants.

The type species is *Rhizobium leguminosarum* Frank.

The name of this genus has been a source of confusion. The organism of leguminous nodules was placed in the mold genus *Schinzia* by Frank as *Schinzia leguminosarum.* Schroeter (1886, p. 134) concluded the organism to be one of the slime molds and created the genus *Phytomyxa,* including it in the order *Phytomyxini* among the *Myxomycetes.* He based his conclusions as to the position of this organism among the slime molds upon the work of Prilleaux (1879, p. 98). Beijerinck (1888, p. 758) named the organism *Bacillus radicicola.* Frank (1889, p. 338) renamed the organism *Rhizobium leguminosarum.* The fact that Schroeter included this genus incorrectly among the slime molds does not invalidate the name. The Committee on Classification of Bacteria, of the Society of American Bacteriologists, however, has recommended the use of the generic name *Rhizobium* as better known and probably resulting in less confusion than the use of *Phytomyxa.*

Genus 3. **Azotobacter** Beijerinck, 1901d, p. 561

Relatively large rods, or even cocci, sometimes almost yeast-like in appearance, dependent primarily for growth energy upon the oxidation of carbohydrates; obligate aërobes, usually growing

in a film upon the surface of the culture medium. Capable of fixing atmospheric nitrogen in considerable amounts when grown in solutions deficient in combined nitrogen. Motile or non-motile, if the latter, with polar flagella.

The type species is *Azotobacter chroöcoccum* Beijerinck.

It is possible that the demonstration of endospore production by Löhnis and his co-workers will require the removal of this genus to the *Bacilleae.*

Subtribe IV. **Bacteriinae** Subtrib. nov.

Cells not fusiform, rod shaped; not hemoglobinophilic; aërobic, facultative, or microaërophilic; not securing growth energy exclusively by the oxidation of carbonaceous compounds. Spores never formed. Motile or non-motile.

The following key to the genera recognized gives the most important differential characters:

Key to genera of Bacteriinae

A. Producing usually a yellowish or greenish or fluorescent pigment, usually Gram-negative, motile by means of polar flagella, or non-motile.
Genus 1. *Pseudomonas*

B. When pigmented not greenish or fluorescent, when motile with peritrichous flagella.
- 1. Cells typically pigmented, chromoparous.
  - a. Producing red or pink pigment..............Genus 2. *Serratia*
  - b. Producing a violet pigment........Genus 3. *Chromobacterium*
- 2. Cells not typically definitely pigmented, or at least not red or violet.
  - a. Cells typically Gram-negative.
    - (1) Non-motile, showing bipolar staining commonly. Never produce gas from carbohydrates. Power of acid production low........................Genus 4. *Pasteurella*
    - (2) Not showing bipolar staining.
      - (a) Not producing honey-like growth on potato, branching forms uncommon.
        - x. Gelatin not liquefied or liquefied very slowly. Motile or non-motile.....Genus 5. *Bacterium*
        - xx. Gelatin liquefied quickly. Motile. Genus 6. *Proteus*
      - (b) Producing a honey-like growth on potato. Branched cells not uncommon. Genus 7. *Pfeifferella*

b. Cells typically Gram-positive. All non-motile.
   (1) Usually microaërophilic. Not typically growing well on the surface of laboratory media. Without metachromatic granules.
      (a) Non-pathogenic lactic acid bacilli. Genus 8. *Lactobacillus*
      (b) Pathogenic, slender, small rods, not lactic acid formers................Genus 9. *Erysipelothrix*
   (2) Aërobic rods, frequently showing metachromatic granules or irregular staining...Genus 10. *Corynebacterium*

Genus 1. **Pseudomonas** Migula, 1894, p. 237, emended

Synonyms:

Bacterium Ehrenberg emended Cohn, 1872, p. 167
Bactrillum Fischer, 1895, p. 139
Arthrobactrinium Fischer, 1895, p. 139
Arthrobactrillum Fischer, 1895, p. 139
Bactrinius Kendall, 1902, p. 484
Bactrillius Kendall, 1902, p. 484
Denitromonas Jensen, 1909, p. 314
Liquidomonas Jensen, 1909, p. 332

Rod-shaped bacteria, never spiral, usually motile by means of polar flagella or rarely non-motile. Aërobic and facultative. Frequently liquefying gelatin. Without spores. Gram stain variable. Usually producing a water-soluble pigment which diffuses through the medium as a green, blue, purple, or brown or in some cases a yellow pigment. Fermentation of carbohydrates usually not active.

The type species is *Pseudomonas aeruginosa* (Schroeter) Frost?

It is difficult to determine whether the generic names *Pseudomonas* or *Bacterium* should be used here. There is evidence that the latter is historically valid for this genus, but it has not generally been so accepted. The generic name *Bacterium* has been used in some seven different senses.

1. Bacterium. The conception held before the work of Cohn, 1872. Relatively rigid cells or chains of cells, not flexible, motile, motion usually oscillatory. The name was used in this sense by Ehrenberg (1828), Dujardin (1841), Perty (1852) and Davaine (1868).

2. Bacterium. Short cylindric or elliptic cells, never in chains or filaments, showing stages of motility and non-motility. Cohn (1872b, 1875), Dallinger and Drysdale (1875), Magnin (1878), Winter (1879), Luerssen (1879), Grove (1884), Van Tieghem (1884), Flügge (1886).

3. Bacterium. Rod-shaped organisms which do not produce endospores. Motility and cell groupings not emphasized. The genus is contrasted with *Bacillus* in these characters. Zopf (1885), Hueppe (1885), Schroeter (1886), De Toni and Trevisan (1889), Ludwig (1892), Freudenrich (1894), Lehmann and Neumann (1896), Löhnis (1913).

4. Bacterium. Not used as a generic name, all rod-shaped organisms being placed in *Bacillus* or other genera. Baumgarten (1890), Sternberg (1892), Fischer (1897), Matzuschita (1902), Schneider (1912).

5. Bacterium. Non-motile rod-shaped organisms. Migula (1895, 1897, 1904), Chester (1901), Smith (1902), Kendall (1902), Ellis (1909), Heim (1911).

6. Bacterium. Polar flagellate organisms of the type of the fluorescent bacilli. E. F. Smith (1906), Vuillemin (1913).

7. Bacterium. Organisms belonging to the colon-typhoid group. Orla Jensen (1909a).

This diversity in definition of this genus has led to considerable confusion in literature. It would seem logical to select one of the species early named *Bacterium,* and designate it as the generic type. However, of the species named before Cohn's descriptions in 1872 there does not seem to be a single one which can be identified with certainty.

It might seem best to select some organism early named *Bacterium* as the type and so word the generic diagnosis as to include this form and the related species.

E. F. Smith has attempted to identify the *Bacterium termo* of Cohn, evidently intended by this author to constitute the type of the genus. He states

His (Cohn's) *Bacterium termo* was a small schizomycetous organism capable of growing freely in Cohn's nutrient solution, containing acid potassium phosphate and ammonium tartrate. It produced thereon

short rods (single, in pairs or fours, joined end to end) and roundish lobed white zoogloeae, together with a *greenish* fluorescence. It did not appear in boiled fluids, i.e. was destitute of endospores (Cohn) and the motile rods were killed by short exposure to 58°C. (Schröeter). In other words, it was a non-sporiferous green fluorescent organism possessed of a single polar flagellum, or in some cases, perhaps, provided with paired or triple polar flagella.

Smith attempted to follow Cohn's procedure in getting cultures of *Bact. termo* and succeeded, by inoculating Cohn's solution with water in which beans had been thrown, in securing a green fluorescent organism with a polar flagellum. He concludes that the morphologically similar non-fluorescent forms and the yellow bacteria should be included in this genus.

Vuillemin by an entirely distinct line of reasoning came to a similar conclusion. He notes that Ehrenberg based his generic description in part upon the type of cell motility, oscillatory. Today, he states, we know this characteristic type of motion to be due to the presence of polar flagella on short rods. He concludes that the *Bact. termo* is closely related to *Bacillus pyocyaneus* and accepts this as the type of the genus.

Still a third line of evidence will lead to a similar conclusion, i.e., a study of the species of *Bacterium* other than *Bact. termo* recognized by Cohn and his coworkers. Three species previously described were placed in the genus *Bacterium* by Schröeter. One of these was the *Vibrio syncyaneus* of Ehrenberg, the organism repeatedly described in literature as the cause of blue coloration in milk. It is a polar flagellate rod. The name *Bact. aeruginosum* is the earlier name applied by Schröter to the blue pus organism, chosen by Vuillemin to constitute the generic type. The organism termed *Bact. xanthinum*, however, belongs to an entirely distinct group.

It is evident therefore that there is historical justification for E. F. Smith's emendation of the genus. The principal criticism is the undue insistence upon motility. It would seem that an organism morphologically similar but non-motile, showing the same fluorescence and physiological characters should likewise be included in the genus.

Notwithstanding the force of the above arguments in favor of using the generic name *Bacterium* for this group, this use will lead to the greatest confusion in the literature. It would seem best to use the name *Pseudomonas* for this genus, designating *Bacterium* as a *genus conservandum* for the colon-typhoid group of bacteria in accordance with the proposal of Orla Jensen (1909a p. 343). This is the recommendation of the Committee on Classification of the Society of American Bacteriologists.

Genus 2. **Serratia** Bizio, 1823, emended

Synonyms:

Zaogalactina Sette, 1824, p. 51
Erythrobacillus. Fortineau, 1905, p. 104

Cells rod-shaped, without spores. Motile by means of peritrichous flagella or non-motile. Gram stain variable. Aerobic, producing a red or pink pigment, a lipochrome. Possibly closely related yellow and orange lipochrome-forming bacteria should be included here as well.

The type species is *Serratia marcescens* Bizio, the organism usually termed *Bacillus prodigiosus.* The synonomy of this organism is as follows:

Serratia marcescens Bizio, 1823
Zaogalactina imetropha Sette, 1824, p. 51
Monas prodigiosa Ehrenberg, 1848
Palmella prodigiosa Montagne, p. 727
Micrococcus prodigiosus Cohn, 1872a, p. 153
Bacillus prodigiosus Fluegge, 1886, p. 284
Bacillus imetrophus Trevisan, 1887
Bacillus marcescens De Toni and Trevisan, 1889, p. 976

Pfeiffer (1887, p. 46) lists *Serratia* under *Fungi dubiae sedis* and ascribes the genus to *Bergamaschi.*

Vuillemin (1913, p. 521) concluded that although the characters used by Bizio have no generic value, nevertheless the generic name might well be revived to include the rod-shaped bacteria with diffuse flagella. He terms *Serratia subtilis* (*Bacillus subtilis*) the type of his emended *genus conservandum.*

Genus 3. **Chromobacterium** Bergonzini, 1881, p. 153

Synonyms. The name was spelled *Cromobacterium* by Bergonzini, and corrected by Zimmerman (1881 p. 1528). The spelling *Chromobacterium* has been accepted by other writers. (Grove, 1884, p. 26; De Toni and Trevisan, 1889, p. 978).

Rod-shaped bacteria, without spores, aerobic, producing a violet chromoparous pigment soluble in alcohol but not in chloroform, motile or non-motile, Gram stain variable.

The type species is *Chromobacterium violaceum* Bergonzini.

Genus 4. **Pasteurella** Trevisan, 1887, p. 94

Synonyms:

Octopis? Trevisan, 1885, p. 102
Coccobacillus Gamaleia, 1888, p. 167
  not *Coccobacillus* Leube, 1885
Dicoccia? Trevisan, 1889, p. 1034
Diplobacillus? Weichselbaum, 1887, p. 212

Short rods, single or rarely in chains, usually showing distinct polar staining, non-motile. Gram-negative, without spores, aerobic and facultative, usually not producing gas, powers of fermentation slight, often pathogenic, not acid-fast, not liquefying gelatin.

The type species is *Pasteurella choleraegallinarum* (Fluegge) Trevisan.

Genus 5. **Bacterium** Ehrenberg, 1828, emended, Jensen, 1909

Synonyms:

Actinobacter Duclaux, 1882, p. 110
Klebsiella Trevisan, 1885, p. 94
Kurthia? Trevisan, 1885, p. 92 in part
Pneumobacillus? Arloing, 1889, p. 428
Pyobacillus Koppanyi, 1907, p. 429
Aërobacter Beijerinck, 1900, p. 198
Salmonella Lignieres, 1900a, p. 389
Tyrothrix? Duclaux, 1879, in part?

Plump rods, without spores, Gram-negative, motile by means of peritrichous flagella, or non-motile, liquefying gelatin very slowly or not at all. Usually showing marked power to ferment carbohydrates, frequently with gas production.

The type species is *Bacterium coli* Escherich.

The genus is a large one, including many species. It is generally divided into subgroups based primarily upon the fermentative reactions. It may be convenient to recognize these as subgenera separated from each other by the characteristics noted in the following key:

Key to the subgenera of Bacterium

A. Organisms which show a maximum of fermentative power, including fermentation of lactose, rarely pathogenic, some forms slowly liquefy gelatin. Subgenus 1. *Aërobacter* (*or Eu-Bacterium*).

B. Organisms not showing maximum fermentative power, never producing gas in lactose, frequently pathogenic, never liquefying gelatin.
   1. Producing acid and gas from glucose, sometimes other sugars, but not from lactose..................... Subgenus 2. *Salmonella*
   2. Producing gas from none of the carbohydrates, acid sometimes formed.................................. Subgenus 3. *Eberthella*

Subgenus 1. **Aërobacter** Beijerinck, 1900, p. 198

Fermenting both glucose and lactose with formation of both acid and gas. Pathogenicity slight.

Type species is *Bacterium* (*Aerobacter*) *coli* Escherich.

Subgenus 2. **Salmonella**, Lignicres,

Fermenting glucose but not lactose with formation of acid and gas. Frequently pathogenic.

The type species is *Bacterium* (*Salmonella*) *cholerae suis*?

Subgenus 3. **Eberthella** subgen. nov.

Not producing gas from any of the carbohydrates, acid may or may not be formed.

The type species is *Bacterium* (*Eberthella*) *typhi*. Flügge.

Genus 6. **Proteus** Hauser, 1885, p. 1

Synonyms:
Liquidobacterium Jensen, 1909a, p. 337
Spirulina Hueppe, 1886, p. 146
not *Spirulina*, Turpin, 1827

Short rods, showing great variation in morphology, filamentous and bent rods as involution forms frequent. Motile by means of peritrichous flagella. The species commonly produce motile "islands" on the surface of moist solid media. No spores. Gram-negative. Usually liquefying gelatin rapidly in absence of carbohydrates. Usually producing acid and gas from certain carbohydrates. In general the species are closely associated with decay and putrefaction, sometimes pathogenic.

The type species is *Proteus vulgaris* Hauser.

Genus 7. **Pfeifferella** gen. nov.

Non-motile rods, slender, Gram-negative, without spores, staining poorly, sometimes forming threads and showing a tendency toward branching. Gelatin may be slowly liquefied. Do not ferment carbohydrates. Growth on potato characteristically honey-like.

The type species is the glanders bacillus, *Pfeifferella mallei.*

Genus 8. **Lactobacillus** Beijerinck, 1901, p. 214

Synonyms:
Lactobacter Beijerinck
Brachybacterium Troili Petersson, 1903, p. 138 in part
Caseobacterium Jensen, 1909a, p. 478
Dispora? Kern, 1882a, p. 135
Tyrothrix? Duclaux, 1882, in part

Rod-shaped organisms, cells frequently quite elongate, non-motile, without spores, Gram-positive in young cultures. Produce acid, largely lactic, from carbohydrates. When gas is produced, it is CO_2 without hydrogen. For the most part the organisms are thermophilic. Microaërophilic.

The type species is *Lactobacillus caucasicus (Kern) Beijerinck.*

Genus 9. **Erysipelothrix** Rosenbach, 1909, p. 367

Rod-shaped organisms with a tendency to the formation of long filaments which may show branching. The filaments may also thicken and show characteristic granules. No spores. Non-motile. Gram-positive. Does not produce acid. Microaërophilic. Usually parasitic.

The type species is *Erysipelothrix rhusiopathiae* (*E. porci*), the causal organism of swine erysipelas.

Genus 10. **Corynebacterium** Lehmann and Neumann, 1896

Synonyms:
Corynemonas Jensen, 1909, p. 22
Corynethrix Bongert, 1901, p. 449

Rods which stain interruptedly (striped) with weak staining solutions. Not acid-fast. Clubbed, wedge-shaped and pointed rods frequent. Gram-positive. Non-motile. No spores. Aërobic.

The type of the genus is *Corynebacterium diphtheriae* Lehmann and Neumann.

Tribe III. **Mycobacterieae** Trib. *nov.*

Synonyms:
Coccothrichaceen Lutz, 1886, p. 22
Mycobacteriaceae Chester, 1897, p. 62 in part

Rod-shaped organisms occasionally showing a tendency to branching, acid-fast, Gram-positive, non-motile, without spores.

A single genus is included, *Mycobacterium* Lehmann and Neumann.

Genus 1. **Mycobacterium** Lehmann and Neumann, 1896, p. 363

Synonyms:
Coccothrix Lutz 1886, p. 22
Sclerothrix Metschnikoff, 1888, p. 70
not *Sclerothrix* Kuetzing, 1849, p. 319

Slender rods which stain with difficulty, but when once stained, are acid-fast. Clubbed, swollen, clavate or cuneate cells occur, even filaments with branches. Non-motile. Without spores. Gram-positive.

The type species is *Mycobacterium tuberculosis* (Koch) Lehmann and Neumann.

Vuillemin (1913 p. 527) contends that *Coccothrix* resembles too closely *Coccotrichum* Wallroth. It does not seem that the resemblance is close enough ever to cause confusion, and the genus name *Coccothrix* is apparently valid. The Committee on Classification of the Society of American Bacteriologists, however, has recommended the use of the generic name *Mycobacterium* as better known and less likely to result in confusion.

REFERENCES

Arloing, S. 1889 Sur l'étude bacteriologique des lesions de la peripneumonie contagieuse du boeuf. Compt. rend. hebd. des Seances de l'Academie des Sciences, **109**, 428.

Babes, V. 1890 *In*, Les Bacteriees, Cornil, A. V. and Babes, V., **1**, 155.

Baumgarten, Paul V. 1890 Lehrbuch der pathologischen Mykologie. Leipzig, 127.

Beijerinck, M. W. 1889 Le *Photobacterium luminosum*. Bacterie lumineuse de la mer du nord. Arch. Neerlandaises des Sciences exact. et nat., **23**, 401.

Beijerinck, M. W. 1893 Ueber die Butylalkoholgärung und das Butylferment. Verhandl. der Konmklijke Akademie van Wetenschappen te Amsterdam, Sect. II, Deel I, No. 10.

Beijerinck, M. W. 1894 Ueber die Butylalkoholgärung und das Butylferment. Abs. Cent. f. Bakt., **15**, 171.

Beijerinck, M. W. 1900 Schwefelwasserstoffbildung in den Stadtgräben und Aufstellung der Gattung Aërobacter. Cent. f. Bakt., II, Abt. **6**, 193–206.

Beijerinck, M. W. 1901a Sur la formation de l'hydrogen sulfure dans les canaux et le genre nouveau Aërobacter. Arch. Neerl. Sci. Holl., Ser. 2, **4**, 1–18. Haarlem.

Beijerinck, M. W. 1901b Sur les Ferments Lactiques de l'Industrie. Arch. Neerlandaises des Sciences Exact. et Nat., Ser. 2, **6**, 213.

Beijerinck, M. W. 1901c Anhaufungsversuche mit Ureumbakterien, Ureumspaltung durch Urease und durch Katabolismus. Cent. f. Bakt., II Abt., **7**, 33.

Beijerinck, M. W. 1901d Ueber oligonitrophile Mikroben. Cent. f. Bakt., II Abt., **7**, 561–582.

BEIJERINCK, W. M. 1904 Ueber die Bakterien welche sich im Dunkeln mit Kohlensäure als Kohlenstoffe ernähren können. Cent. f. Bakt., II Abt., **11**, 598.

BERGONZINI, CURZIO 1881 Sopra un nuovo Batterio Colorato. Annuario della Societa dei naturalisti in Modena. Ser. 2, **14**, 149–158.

BIEDERT, PH. 1885 Beitrag zur Frage nach der Constanz der Spaltpilze. (*Kokkobacillus zymogenus* and *Bacterium termo.*) Virchow's Arch., **100**, 439.

BILLET, A. 1890 Contribution a l'étude de la Morphologie et du Developpement des Bacteriacees. Extrait du Bulletin scientifique de la France et de la Belgique. Paris.

BILLROTH, THEODOR 1874 *Coccobacteria septica.*

BIONDI, D. 1887 Die pathogenen Mikroorganismen des Speichels. Ztschr. f. Hyg., **2**, 192–236.

BIZIO, B. 1823 Polent. porporp. Bibl. ital., **30**, 288.

BONGERT 1901 Corynethrix pseudotuberculosis murium ein neuer pathogener Bacillus für Mäuse. Ztschr. f. Hyg., **37**, 449–475.

BORREL, DUJARDIN-BEAUMETZ, JEANTET AND JOUAN. 1910 Le Microbe de la Peripneumonie. Annales de l'Inst. Pasteur, **24**, 179.

CHATTON AND PERARD 1913 Schizophytes du coecum du cobaye. II Metabacterium polyspora n. g. n. sp. compt. rend. hebd. des seances et mem. de la Soc. de Biologie, **65**, 1232.

CHESTER, F. D. 1897 Classification of the Schizomycetes. Ninth Annual Report Delaware College Agr. Exp. Sta., pp. 62–63.

CHESTER, F. D. 1901 Manual of determinative bacteriology.

COHN, FERDINAND 1854 Untersuchungen über die Entwickelungsgeschichte der mikroskopischen Algen und Pilze. Der Akademie übergeben den 1 Mai, 1853. Nov. Act. Acad. Caes. Leop.-ü Carol. Nat. Cur., **24**, 103–256.

COHN, FERDINAND 1872a Organismen in der Pockenlymphe. Virchow's Arch., **55**, 229–238 (a).

COHN, FERDINAND 1872b Untersuchungen über Bakterien. Beiträge zur Biologie der Pflanzen, **1** (Heft 1), 127–224 (b).

COHN, FERDINAND 1875 Untersuchungen über Bakterien. II Beiträge z. Biologie d. Pflanzen, **1** (Heft 3), 141–208.

CONN, H. W. 1909 Agricultural Bacteriology, 2 ed., p. 12.

CORNIL AND BABES 1890 Les Bacteries.

DALLINGER, W. H. AND DRYSDALE, J. J. 1875 On the existence of flagella in *Bact. termo*. Monthly Microscopical Journal, p. 105.

DANGEARD, A. 1891 Contribution a l'étude des Bacteriacees vertes (Eubacillus nov. gen.). Le Botaniste, **2**, 151.

DAVAINE, C. 1868 Dictionnaire Encyclopedique des Sciences Medicales, Ser I, **8**, 21.

DE BARY, A. 1884 Vergl. Morph. u. Biol. d. Pilze. Mycetozoa und Bacterien. Leipzig.

DENNSTÄTT 1818, (according to Schroeter, l. c., p. 134)

DETONI, J. B. AND TREVISAN, V. 1889 Schizomycetaceae Naeg. in Saccardo's Sylloge Fungorum. **8**, 923–1087.

DUCLAUX, E. 1882–1883 Deuxieme memoire sur le lait. Ann. de l'Inst. Agron., **7**, 35.

Dujardin, F. 1841 Histoire naturelle des Zoophytes. Paris.
Ehrenberg, C. G. 1828 Symbolae Physicae, Animalia Evertebrata.
Ehrenberg, C. G. 1848 Berichte über die Verhandlungen der Königliche Preuss. Akademie zu Berlin, 349, 354, 384, 462.
Ehrenberg, C. G. 1849 Ibid., 101.
Ehrenberg, C. G. 1850 Ibid., 364.
Ellis, David 1909 Outlines of Bacteriology, p. 6.
Fick 1887 Ueber Mikroorganismen im Conjunctivalsack. Wiesbaden.
Fischer, Alfred 1895 Untersuchungen über Bakterien. Jahresb. f. wiss ensch. Bot., **27**, 1–163.
Fischer, Alfred 1903 Vorlesungen über Bakterien. 2d ed.
Flügge, C. 1908 Grundriss der Hygiene, Ed. 6, p. 514.
Forti, C. 1896 Notizie complementari su alcuni studi di zimotecnia enologica esegwiti a tutto il. Ann. della soc. chim. di Milano, vol. **3**, fasci. 1.
Fortineau, L. 1905 *L'Erythrobacillus pyosepticus*. Compt. rend. Soc. biol., **58**, 104.
Frank, B. 1879 Ueber die Parasiten in den Wurzelausschwellungen der Papilionaceen. Bot. Zeitung **37**, 376.
Frank, B. 1889 Ueber die Pilzsymbiose der Leguminosen. Ber. d. deut. bot. Gesellsch. **7**, 338.
Freudenreich 1894 Les Microbes et leur role dans la laiterie.
Fuhrmann, F. 1905 Morphologisch.-biologische Untersuchungen über ein neues Essigsäuresbildendes Bakterium. Beiheft zum. bot. Centralbl. **19**, 1–31.
Gamaleïa, N. 1888 Zur Aetiologie der Hühnercholera. Centralbl. f. Bakt. **4**, 167.
Gonnermann, M. 1907 Ueber gallertbildende Bakterien. Oesterungar. Ztschr. f. Zuckerind. u. Landwirtsch. **36**, 877–888.
Grove, W. B. 1884 A Synopsis of the Bacteria and Yeast Fungi.
Hartlieb, R. 1900 Die Morphologie und systematische Stellung der sogenannten Knöllchenbakterien. Chem. Zeitung. **24**, 887.
Hauser 1885 Fäulnisbakterien.
Heim, Ludwig 1911 Lehrbuch der Bakteriologie, 4 ed. p. 250.
Hewlett, R. I. 1898 A Manual of Bacteriology, p. 18. Philadelphia.
Hoelling 1910 Die Kernverhältnisse von *Fusiformis termitidis*. Arch. f. Protistenkunde **19**, 240.
Hueppe, F. 1886 Die Formen der Bakterien und ihre Beziehungen zu den Gattungen und Arten. Wiesbaden.
Itzigsohn 1867 Sitz. Ges. nat. Fr. zu Berlin. Novem. According to Koch in Cohn's Beiträge II, 3, p. 414.
Jensen, Orla 1909a Die Hauptlinien des natürlichen Bakteriensystems. Centralbl. f. Bakt., II Abt., **22**, 305–346.
Jensen, Orla 1909b Vorschlag zu einer neuen bakteriologischen Nomenklatur. Centralbl. f. Bakt., II Abt., **24**, 477.
Kendall, Arthur I. 1902 A Proposed Classification and Method of Graphical Tabulation of the Characters of Bacteria. Public Health. **28**, 484.
Kern, Edward 1882a Dispora caucasica. Nov. gen. et. nov. sp. eine neue Bakterienform. Biol. Zentralblatt **2**, 135.

KERN, EDWARD 1882b Ueber ein neues Milchferment aus dem Kaukasus. Bull. de la Soc. Imperiale des Naturalistes de Moscou. 2d Part, **56**, 141–173.

KIRCHNER, O. 1895 Die Wurzelknöllchen der Sojabohne. Cohn's Beitr. zur Biol. d. Pflanzen **7**, 213.

KLEBS, E. 1871 Die Ursachen der Infectiösen Wundkrankheiten. Correspondenzbl. f. Schweizer Aerzte. ?

KOPPANYI, EMERICH 1907 Ueber eine mit fibrinösen Pleuritis einhergehende Pyämie der Kaninchen. Ztschr. f. Tiermed. Jena. **11**, 429–454.

KÜTZING, FRIEDRICH 1904 Mikroskopische Untersuchungen über die Hefe und Essigmutter, nebst mehreren anderen dazu gehörigen vegetabilischen Gebilden. Auszuge vorgetragen in der Versammlung des naturhistorischen Vereins des Harzes in Alexisbad am 26 Juli 1837. Reprinted Delbrück, M. and Schrohe, A. Hefe, Gärung und Fäulnis. pp. 25–38.

KÜTZING, F. T. 1849 Species Algarum.

LEHMANN, K. B. AND NEUMANN, R. 1896 Atlas und Grundriss der Bakteriologie München, vol. 1 and 2.

LEUBE 1889 *In* Virchows Arch. B. C. fasc. 3. 1885. According to Saccardo, **8**, 966.

LÖHNIS, F. 1913 Vorlesungen über landwirtschaftliche Bakteriologie, p. 45.

LOTSY, J. P. 1907 Vorträge über botanische Stammesgeschichte. Vol. I, Algen und Pilze.

LUDWIG, FRIEDRICH 1892 Lehrbuch der niederen Kryptogamen.

LUERSSEN, CHR. 1879 Botanik. **1**, 22.

LUTZ, ADOLPH 1886 Zur Morphologie des Mikroörganismus der Lepra. Separat Abdruck aus Dermatologische Studien. Herausg. von Dr. P. G. Unna. Heft. 1, p. 22.

MAASSEN, ALBERT 1905 Ueber Gallertbildungen in den Säften der Zuckerfabriken. Ein Beitrag zur kenntniss der gallertbildenden Bodenbakterien. Arb. a. d. Kaiserl. Gesundheitsamte. Biol. Abt. **5**, 1.

MACE, E. 1897 Traité pratique de Bacteriologie, 3d ed.

MAGGIO (RIVOLTA?) 1887 L'Allevatore, No. 1.

MAGNIN, A. 1878 Les Bacteries. Paris. English translation by G. M. Sternberg, 1880.

MALERBA, P. AND SANNA-SALARIS, G. 1888 Ricerche sul Gliscrobatterio. Rendiconto della R. Accademia delle Science fis. e. nat. di Napoli. Fasc. 6. According to Buchner. Centralbl. f. Bakt. **4**, 486.

MATZUSCHITA, T. 1902 Bakteriologisches Diagnostik.

METSCHNIKOFF, ELIAS 1888 Ueber die phagocytäre Rolle der Tuberkelriesenzellen. Virchow's Arch. **113**, 63–94.

MEYER, ARTHUR 1898 Neues über die Morphologie der Bacterienzelle und die Entwickelungsgeschichte der Bacteriensporen. Sitzungsberichte der Gesellschaft zur Befördung der gesammten Naturwissenschaften zu Marburg, No. 5, p. 49, July 1897.

MIGULA, W. 1894 Ueber ein neues System der Bakterien. Arb. a. d. bakt. Instit. d. Technisch. Hochschule zu Karlsruhe. **1**, 235–238.

MIGULA, W. 1895 Schizomycetes. *In* Engler and Prantl Pflanzenfamilien, p. 20.

MIGULA, W. 1897, 1900 System der Bakterien. Vol. 1, 1897, vol. 2, 1900.

MIGULA, W. 1904 Allgemeine Morphologie. Entwickelungsgeschichte Anatomie und Systematik der Schizomyceten, in Lafar's Handbuch der technischen Mykologie, 2d ed, pp. 1–149.

MILLER, W. D. 1886 Einige gasbildende Spaltpilze des Verdauungstractus, ihr Schicksal im Magen und ihre Reaction auf verschiedene Speisen. Deut. Med. Wochschr. **12**, 117–119.

MIQUEL, M. P. 1879, 1880, 1881 Etudes sur la fermentation ammoniacale et les ferments de l'uree. Ann. de Micrographie. **1**, 414, 417, 506, 552. 1879. **2**, 13, 53, 122, 145, 367, 488. 1880. **3**, 275, 305. 1881.

MORREN, C. F. A. 1830 Bull des Scienc. natur. n. XXVII.

MUELLER, O. F. 1786 Animalcula infusoria et marina, Hauniae.

NAEGELI, CARL WILHELM VON 1842 Botanische Beiträge. Linnaea **16**, 279.

NAEGELI, CARL WILHELM VON 1849 Gattungen einzelliger Algen, physiologisch and systematisch bearbeitet. Zurich.

PERSOON 1822 Mycologia europeae. Sectioprima, p. 96.

PERTY, MAXIMILIAN 1852 Zur Kenntniss kleinster Lebensformen, Bern.

PFEIFFER 1887 Synonymia botanicae, p. 46.

PRAZMOWSKI, ADAM 1880 Untersuchungen über die Entwickelungsgeschichte und Fermentwirkung einiger Bakterien, p. 23, Leipzig.

PRILLIEUX 1879 Sur la nature et la cause de la formation des tubercules qui naissent sur les racines des Legumineuses. Bull Soc. Bot. de France. **26**, 98.

RIVOLTA, SEBASTIANO 1888 Pleurite infettiva nel gatto e nel cane. Giornale di Anatomia Fisiologia e Patologia degli Animali, **20**, 1–8.

ROSENBACH, F. J. 1909 Experimentelle, morphologische und klinische Studien über die krankheitserregenden Mikroörganismen des Schweinerothlaufs, des Erysipeloides und der Mäusesepsis. Ztschr. f. Hyg. **63**, 343–371.

ROTHMANN, E. A. 1904 Glischrobacterium als Ursache der schleimigen Gärung des Menschurins. Centralbl. für. Bakt. I, Abt. Orig., **37**, 491.

SCHNEIDER, A. 1912 Pharmaceutical Bacteriology.

SCHRÖTER, J. 1886 Die Pilze, in Cohn, Kryptogamen-Flora von Schlesien.

SCHRÖTER, J. 1889 Phytomyxinae in Engler and Prantl Nat. Pfl. fam. **I**, **1**, 7.

SETTE, VINCENZO 1824 Sul l'arrossimento straordinario di alcune sostanze alimentose osservato nella provincia di Padova l'anno 1819. Venezia p. 51.

SMITH, ALLAN J. 1902 Laboratory Exercises in Bacteriology, p. 270.

SMITH, ERWIN F. 1905 Bacteria in relation to plant diseases. Vol. 1. Carnegie Institution, Washington, D. C.

STERNBERG, G. M. 1892 Manual of Bacteriology, N. Y.

TRECUL, A. 1865 Production de plantules amyliferes dans les cellules vegetales pendant la putrefaction. Chlorophylle cristallisee. Compt. rend. hebd. de seances de l'Acad. de Paris. **61**, 432–436.

TREVISAN, V. 1879 Prime linee d'introduzione allo studio dei Batterj italiani. Rendiconti. Reale Instituto Lombardo di Scienze e Lettere IV. Series II, **12**, 133–151.

TREVISAN, V. 1885 Caratteridi alcuni nuovi generi di Batteriacee Atti della Accademia Fisio-Medico-Statistica in Milano. Series 4. **3**, 92–107.

TREVISAN, V. 1887 Se sia vero che il Bacillo imetrofo (Micrococcus prodigiosus di antichi autori) preceda sempre come agente specifico predisponente il calcino del baco da seta. Rendiconti. Reale Istituto Lombardo di Scienze. Lettere. Series II. **20**, 797–804.

TREVISAN, V. 1889 Genera. E sp. delleBatteriacee. According to De Toni, J. B. and Trevisan, V. Schizomycetaceae Naeg. *in* Saccardo. Sylloge Fungorum **8**, 927.

TROILI-PETERSON, GERDA 1903 Studien über die Mikroörganismen des schwedischen Güterkases. Centralbl. f. Bakt., II Abt., **11**, 138.

VAN LAER, H. 1889 Contributions a l'histoire des ferments des hydrates de carbone. Bacille des kieres tournees. Mem. de l'Acad. royale de Belgique. **43**.

VAN TIEGHEM, PH. 1884 Traité Botanique. Paris, p. 1114.

VUILLEMIN, PAUL 1888 Les tubercules radicaux des legumineuses. Ann. de la Sci. Agron. Franc. et Etrangére. Vme. Ann. T. I., pp. 121–212.

VUILLEMIN, PAUL 1913 Genera Schizomycetum. Annales Mycologici **11**, 526.

WEICHSELBAUM 1887 Centralbl. f. Bakt., **2**, 212.

WINTER, GEORG 1880 Die Pilze, in:—Rabenhorst's Kryptogamen Flora, 2 ed. Vol. 1.

ZIMMERMAN 1880 Review of Bergonzini on Cromobacterium. Bot. Centralbl. **4**, 1528.

ZOPF. W. 1883, 1885 Die Spaltpilze Ed. 1, 1883. Ed. 3, 1885.

A STUDY OF GREEN FLUORESCENT BACTERIA FROM WATER[1]

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INTRODUCTION

In all branches of biology a systematic arrangement of the living organisms is essential to good progress. Science has been defined as an exact and systematic statement of knowledge concerning some subject or group of subjects. Such a definition requires that investigations be constantly carried on to incorporate new facts with the old ones into an orderly arrangement. In bacteriology however, the early workers were too busy identifying new forms to give much attention to classification, and when they did turn their attention to this important branch of the science the pleomorphists pointed out the difficulties of systematizing a science the bases of which were constantly changing. It is only in comparatively recent times that the bacteria in water have been given any intensive study of this kind.

HISTORICAL

Classification of water bacteria

The water bacteriologists have been interested primarily in the presence of certain bacteria which are of sanitary significance. They have studied but superficially the large number of other bacteria which may be present. This may have been due

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to the varied character of water bacteria, for the environment of a water determines its bacterial content, and consequently a water flowing from an unpolluted drainage basin will have a different flora from that of a stream which carries the wastes of domestic and industrial life.

With the exception of some ten bacteria or groups of bacteria, whose presence is supposed to indicate pollution in water, the large number of other bacteria which are present are almost unknown. Information in regard to them would often favor more accurate opinions with regard to quality, and more definite information with regard to the source of water. Certain bacterial standards have been formulated which state that there must not be over 100 bacteria per cubic centimeter in a filter effluent. The character of those bacteria which are present in effluents having a greater number would for instance be important in making deductions with regard to the efficiency of the filters.

The group arrangement has been used for some time in treating the flora of a substance like water. Wyatt Johnston (1894) early called attention to this method. He discussed in his paper the tests which should be used to separate bacteria; and thought that a single strongly marked characteristic was more important for grouping than a number of minor points. The American Public Health Association later appointed a committee (1898) to work out uniform methods for the description of bacteria. This is one of the first instances in which the organized efforts of a society were brought to bear in systematizing methods for the study of bacteria.

Ward (1897) studied the flora of the Thames River and arranged the forms which he isolated in 21 groups, 2 of which are made up of fluorescent bacteria. The separation was often made on very unimportant characteristics. Ward realized this and stated that his work indicated wider limits of the term species than are ordinarily assumed. Fuller and Johnson (1899) in studying the flora of river water made an important contribution to water bacteriology. They divided water bacteria into 13 groups, and used some prominent character for the separation

of each. The fluorescent characteristic played an important rôle. Jordan (1903) made 27 groups when studying 543 strains from the Mississippi, Missouri and Illinois rivers. Two of his groups are; V. *B. fluorescens liquefaciens*, VI. *B. fluorescens non-liquefaciens*. Cornwall (1915) classified water bacteria from a sanitary standpoint. He used, however, morphological data which were too limited to allow a classification of wide application.

This grouping of bacteria introduces the question as to what is a "species." Ward pointed out that we may now be considering varieties of the same organism as separate species. Upon this question there is no agreement among bacteriologists. Winslow (1914) summed up the present conception of this question when he stated that "for practical purposes, however, we must recognized certain types as 'species' or 'varieties' even though they may sometimes intergrade."

The descriptive chart of the Society of American Bacteriologists

Bacteriologists for some time have been working out a system for classifying bacteria. The chart of the Society of American Bacteriologists represents a stage in the development of this effort. A history of the development of the card and of the numerical system of recording characters of bacteria has been prepared by Harding (1910), a very brief outline of which is given here.

Doctor Wyatt Johnston (1894) first called attention to the possibility of using some such method as the Dewey numerical system in the classification of bacteria. A committee was appointed which in its reports suggested the beginnings of such a procedure. Conn (1906) adopted these suggestions in the classification of dairy bacteria. The first real attempt to use such a group number as our present one, reported by Gage and Phelps (1902), was made by Kendall at the Lawrence Experiment Station. The genus classification of bacteria proposed by Migula was used as the basis for their chart.

The present chart has a group number depending on the determination of ten characteristics. It has been recently described by Rahn and Harding (1914). This system of record-

ing the characteristics of bacteria has not met with entire approval on the part of all bacteriologists. One of those who was instrumental in introducing this numerical system has said that it was a thing he regretted. Whether it is, or will be, of any value may be determined after it has been used to study various groups as has already been done in a few cases.

Harding (1910) reported the study of the *Pseudomonas campestris* group. For each strain the same group number was obtained which would indicate that the group number was well fitted to these bacteria. This author believes that it may not "carry the separation to a group synonymous with the ordinary conception of species." The statement seems to have been borne out by later investigations.

Harding and Prucha (1908) used the Society's chart in the study of the flora in cheddar cheese. In their conclusions they state that this method of recording the reaction of cultures is a marked advance in technique and that changes in the cheese flora may be traced more accurately by its use. The sum and substance of their opinion of the chart in its application to the study of cheese bacteria is that it is a valuable means to an end.

Harding, Morse and Jones (1909) in their study of soft rot organisms use the group number in studying their strains. By examining their data it is apparent that the group number is very valuable in studying soft rot organisms, as noted by Harding in 1910 when the same group was studied.

Conn (1906) in his classification of dairy bacteria used a group number, but does not draw any definite conclusions with regard to its value. He still retained names for the organisms which he studied.

More recently, H. J. Conn (1915) has studied 130 cultures of *B. subtilis* by means of the society chart. In selecting these cultures one-half of the determinations represented in the group number were fulfilled because they were implied in the definition of *B. subtilis*. His conclusions are that different group numbers do not always represent different species, and that better methods for making these ten determinations should be devised.

Edson and Carpenter (1912) used the group number in study-

ing bacteria in maple sap. No statements seem to have been made with regard to the value of the Society's chart in the study of the sap bacteria.

The numbers used for recording the various physiological reactions according to the 1912 Society's chart are given in table 1. The numbers of the 1914 chart are identical except those used for action on nitrates.

The fluorescent group of bacteria

This has often been considered a heterogeneous group of bacteria having one property in common—that of excreting a green diffusible pigment capable of causing the medium to fluoresce. Kruse (1896) believes that the members of this group have little phylogenetic relationship, but rather fall into widely related groups. These special bacteria are obviously more conspicuous than the non-fluorescent bacteria, and so have been grouped together. By some bacteriologists however, this group has been called a species.

Ruzicka (1898) believes that there are in the fluorescent group bacteria closely related to the semi-pathogenic organism, *B. pyocyaneus* on the one side and *B. fluorescens-liquefaciens* on the other. Some of his data seem to justify this conclusion.

In Jordan's paper on "The Kinds of Bacteria in River Water" the fluorescent bacteria are discussed. He studied 58 strains of these bacteria, 33 of which liquefied gelatin. The power of liquefying gelatin was found to be closely associated with the ability to coagulate milk. In other characters the two kinds were much alike. All of the strains studied by Jordan were short motile rods. Attention is called to the apparent identity of many of the various forms described in the literature. He mentions the names of about 50 fluorescent bacteria at the close of his paper.

A number of questions may be asked with regard to this group of bacteria characterized by a fluorescent pigment. Is there a good reason other than the formation of this green pigment for setting aside these forms into a group? Is this character of

TABLE 1

Numerical system of recording the salient characters of an organism (group number)

100.0	Endospores produced
200.0	Endospores not produced
10.0	Aerobic (strict)
20.0	Facultative anaerobic
30.0	Anaerobic (strict)
1.0	Gelatin liquefied
2.0	Gelatin not liquefied
0.1	Acid and gas from glucose
0.2	Acid without gas from glucose
0.3	No acid from glucose
0.4	No growth with glucose
0.01	Acid and gas from lactose
0.02	Acid without gas from lactose
0.03	No acid from lactose
0.04	No growth with lactose
0.001	Acid and gas from sucrose
0.002	Acid without gas from sucrose
0.003	No acid from sucrose
0.004	No growth with sucrose
0.0001	Nitrates reduced with evolution of gas
0.0002	Nitrates not reduced
0.0003	Nitrates reduced without gas formation
0.00001	Fluorescent
0.00002	Violet chromogen
0.00003	Blue chromogen
0.00004	Green chromogen
0.00005	Yellow chromogen
0.00006	Orange chromogen
0.00007	Red chromogen
0.00008	Brown chromogen
0.00009	Pink chromogen
0.00000	Non-chromogenic
0.000001	Diastasic action on potato starch, strong
0.000002	Diastasic action on potato starch, feeble
0.000003	Diastasic action on potato starch, absent
0.0000001	Acid and gas from glycerol
0.0000002	Acid without gas from glycerol
0.0000003	No acid from glycerol
0.0000004	No growth with glycerol

The genus according to the system of Migula is given its proper symbol which precedes the number thus:

Bacillus coli (Esch.) Mig. becomes	B 222.111102
Bacillus alkaligenes Petr. becomes	B 212.333102
Pseudomonas campestris becomes	Ps 211.333251
Bacterium suicida (Mig.) becomes	Bact. 222.232203

sufficient importance for such a purpose? Such questions may be answered by a detailed examination of individual strains.

Niederkorn (1900) studied 15 strains of fluorescent bacteria in order to find, if possible, better methods for differentiating the various forms. He thinks that there are only two constant forms, *Bacillus pyocyaneus* (Gessard) and *Bacillus fluorescens-liquefaciens* (Flügge) and that the others are varieties. The work of Eisenberg (1914) shows that the group of fluorescent bacteria is closely related. He reports some work which he did on seven strains and finds intergrading characters which make it difficult to separate them into species and varieties.

Buchanan (1915) has very aptly expressed the condition of bacterial nomenclature. His statement is directly applicable to the fluorescent group. "The naming of bacterial species, genera, and higher groups, indeed the whole subject of bacterial nomenclature, is in a condition which can best be described as chaotic." Many different investigators have studied bacteria which are fluorescent and have given new names to varieties which differed slightly in characteristics. A search of the literature revealed 95 different names which have been given to fluorescent bacteria. There is no reason to think that this number includes all of them. One of the most recent additions to the fluorescent group is *Bacillus cereus* variety *fluorescens* nov. var, which has been reported by Laubach (1916).

Significance of the fluorescent group of bacteria

Bacteria having such a strong aromatic odor may have some significance in the industries. Conn (1910) ascribes certain cases of rancidity in butter to the presence of fluorescent bacteria. The possibility of this is apparent to anyone who has extracted the pigment with any of the usual solvents. Even in small amounts the odor remains for a long time in the room in which such extractions have been made.

Griffon (1909) calls attention to the wide distribution of the fluorescent bacteria in nature, and to the fact that they are present in many plant diseases and cause rot in vegetables.

Edson and Carpenter (1912) in their work, mentioned elsewhere, prove that fluorescent bacteria play an important part in the decomposition of maple sap. Thoni (1911) found fluorescent forms in a bacterial study of lemonade. As has been mentioned before these bacteria are abundant in water supplies. The strains which were studied in this paper were all taken from water supplies in Illinois. Schmelch (1888) found fluorescent bacteria in cold glacial waters. Harrison (1898) found these forms in hail, as did Belli (1902).

Routine analysis of samples of water sent to the Illinois State Water Survey indicate that fluorescent bacteria are rather common in waters from the Illinois and Mississippi rivers. They are especially abundant in the samples from the Illinois river which is rather heavily laden with organic matter, indicating that they are more abundant where organic matter is undergoing decomposition.

Previous studies on the fluorescent group of bacteria

The most important investigation of this group, as such, was carried out at the Vermont Agricultural Experiment Station by Edson and Carpenter (1912). These investigators used the society chart in their study of 42 strains of fluorescent bacteria coming from maple sap which was collected from different orchards in Vermont. The paper contains a complete description of the strains with the following group numbers:

12 strains	Pseudomonas	221.2332132	
12 strains	Pseudomonas	221.2332133	
7 strains	Pseudomonas	221.2322132	
4 strains	Pseudomonas	221.2322133	
2 strains	Pseudomonas	221.2333133	
1 strain	Pseudomonas	221.2323132	
2 strains	Pseudomonas	221.2222132	
1 strain	Pseudomonas	221.2232133	
1 strain	Bacillus	221.2222?32	Nov. Sp.

From these group numbers may be inferred the close relationship of the strains. All of them liquefied gelatin, but it was necessary to keep some of the gelatin tubes longer than six

weeks, the period of time recommended on the Society's chart. Seven strains began to liquefy after three to five months. The time for complete liquefaction was not determined, but at the end of eight and one-half months the tubes when melted and poured into plates, showed growth in pure culture. The group number is made up on the assumption that all strains liquefied gelatin. No spore formers were found in any of the strains.

EXPERIMENTAL

Method of study

The descriptive chart of the Society of American Bacteriologists was used for recording the data in this investigation of the fluorescent bacteria. The group number was determined for each strain under as uniform conditions as possible. One objection to this group number is the lack of uniform and satisfactory methods for the determination of some of the digits. Individual workers may use their own methods, and it is possible that group numbers determined in different laboratories ought not to be compared. It is understood that a code of standard methods is being prepared which ought to allow a wider application of the group number in systematic studies of bacteria.

Media and technique

The media and technique used in this study conformed to Standard Methods for the Examination of Water and Sewage of the American Public Health Association, 1912, except that in place of meat infusion, Liebig's meat extract was used. It was believed that in this way more uniform media were secured. In those instances where special media were used the composition will be mentioned under the special heading.

Sources of strains

All the strains of fluorescent bacteria were isolated from samples of water which were collected at different places in the

state of Illinois. It was decided to limit this study to the forms obtained from water, and at a later date, if possible, to study another series from a different source. The investigation by Edson and Carpenter was made with strains many of which came from the same sap orchard. In this way their strains may have had some close relationship. The strains which form the basis of the present study are probably in no way related. The source of these strains is given in table 2.

TABLE 2

Surface waters	35
Shallow wells (under 100 feet in depth)	50
Deep wells (over 100 feet in depth)	3
Springs	4
Cisterns	3
Swimming pool	1
Sewage	1
Water of unknown source	1
Water from interstate carrier	1
Ice	1

The colonies were picked from agar and gelatin plates which had been made from water samples received at the laboratory of the Illinois State Water Survey. All of these samples were collected according to instructions which were furnished with the sterile container. They were shipped to the laboratory packed in ice, the examination was immediately started on receipt of the sample and after the plates had been counted for the routine analysis, they were examined for fluorescent bacteria. If none were evident, it was found that they often appeared after the plates had been held at room temperature over night. Young strains were used in all cases. All inoculations into the various media were made either from a twenty-four hour broth culture or from a twenty-four hour agar slant culture.

Description of strains

Since the Society's chart was used in this study the various cultural and morphological characteristics will be mentioned in somewhat the order in which they appear on the chart.

Morphology of strains

All of the strains studied were motile rods, and usually grew in chains. The cells used in studying the morphology were young and were subcultured on plain agar slants before being stained. The vegetative cells were both long and short rods with rounded ends, some of them being distinguished from coccus forms only with great difficulty. The agar slants were incubated at 37°C. for twenty-four hours, and the study of morphology was made from these smears stained with carbol fuchsin.

The cells were measured by means of a Leitz filar micrometer, but the size was not especially significant with these bacteria.

Only four of the cultures showed spore formation. These were 45, 47, 61, and 70. All of the strains possess a great similarity, as is shown by the following group numbers.

45 and 61	121.2332133
47	121.2333133
70	122.2333133

Culture 70 differs from the other three spore formers in not liquefying gelatin. Nitrate reduction is absent in strains 45 and 61. Three of them are from shallow wells, while 47 comes from the Illinois River at Marseilles. A fluorescent spore forming bacillus from water has recently been described by Laubach (1916). The flagella were not studied and he may have been working with a pseudomonas form.

All of the strains were pseudomonads when Loeffler's method was used for staining the flagella. Most of the cultures possessed a *tuft* of flagella on one end, while those with but one flagellum were rare. The length of the flagella was not measured.

Motility was observed with each strain. A few failed to show it upon first observation, but subsequent examinations, however, gave ample proof of motility.

The staining properties were not especially characteristic. All cultures stained with the ordinary aqueous alcohol stains and were Gram-negative.

Capsules were demonstrated on the following cultures by means of Welch's method as described by Muir and Richie (1913).

2, 3 Ps	221.2223133
6 Ps	221.2232133
14 Ps	221.2332133
22 Ps	221.2222132
26 Ps	221.2233133
37, 75 Ps	221.2333133
46 Ps	221.2332133
50 Ps	221.2223133
60 Ps	222.2223132
68 Ps	222.2233133
79 Ps	222.2223133

The broth cultures of these strains were invariably viscid. The group numbers are quite similar, but differ slightly in a few characteristics. All but three strains liquefied gelatin. These strains gave slimy growth in most media.

Growth on agar stroke

All strains were grown on this medium and produced a distinct fluorescence. Abundant growth was secured at 37°C. One strain secured from another laboratory refused to grow at 37°, but grew well at 20°. This strain, however, was not included. Strain 100 was peculiar in that the fluorescent color was followed by a permanent red. This may have been due to an acid reaction of the medium.

Growth of potato slants

The potato slants were made in the usual way. In almost every case the growth was spreading and was either moderate or abundant. At the end of about two days incubation it was limited to the line of inoculation, but spread rapidly after longer incubation. The medium was turned to a dark brown or green. Observations were made at the end of ten days, when the potato and the water in the bottom of the tube were poured into a mortar and tested for starch destruction.

Starch destruction

No official method for determining the presence of diastase is available, and each worker uses his own favorite technique. The method used by Edson and Carpenter required the addition of 2 per cent thymol starch paste to a ten day old broth culture. After incubation for eight hours the culture was filtered and tested for reducing sugar by means of Fehling's solution.

The method suggested by Smith was used in this work, as outlined by Harding (1910). Cultures of the bacteria were grown on potato slants for ten days at 37°C. At the end of this time these slants were crushed in a mortar together with the water in the bottom of the culture tubes. This mixture was diluted with distilled water and tested with a weak solution of iodine in potassium iodide for the split products of starch. If the starch mixture had not been sufficiently diluted the blue color which this substance gives with iodine would have entirely masked the color given by some of the decomposition products. The presence of erythrodextrin is shown by a red color. Good results may be obtained with this method after one has become accustomed to the color changes which are involved. Starch agar plates were also used. These were made by adding 1 cc. of a sterile 2 per cent solution of soluble starch to plain agar in Petri dishes. Streaks of the pure culture were made upon this medium and the plates incubated at 37° for five days. At the end of this period a dilute solution of iodine in potassium iodide was permitted to flow over the surface of the plate. With those bacteria which decompose starch a colorless area will be noted about the line of growth. None of the cultures studied were able to decompose starch. This is perhaps not to be expected when one recalls the strong proteolytic action of this group. Over 50 per cent of the cultures were able to break down gelatin, as reported by other investigators.

Agar colonies

Colony growth on agar was always abundant. After the colonies were well developed a large amount of soluble pigment was formed. The edge was always irregular and rarely entire.

Gelatin colonies

All cultures gave abundant growth on this medium. The non-liquefiers grew on the surface and often spread over a large area. Fluorescence was very evident on this medium. The liquefying cultures gave large saucer-shaped colonies which were filled with a flocculent growth.

Gelatin stab

The medium used in this test was made from Gold Label French gelatin. Twelve per cent of gelatin was added to plain peptone bouillon. The medium was put into test tubes to the depth of about 7 cm. Inoculations were made into these tubes by means of a platinum needle, stabbing to the bottom of the test tube. The cultures were incubated for thirty days at 20°C., as recommended on the descriptive chart of the Society of American Bacteriologists.

At the end of thirty days 59 of the cultures had liquefied the gelatin. The gelatin cultures of the other 41 were left in the incubator for over fourteen months. During that time strains 7, 19, 67 and 83 liquefied the medium. With strains 7 and 19, the liquefaction took place at the end of three months. Strains 67 and 83 required about six months for this change. The other 37 strains did not liquefy gelatin in fourteen months when kept at 20°C. The tubes were fitted with rubber caps to prevent evaporation. In determining the group number, only those strains were considered as liquefiers which produced liquefaction in thirty days. In all cases the growth seemed to be best at the surface, although it often extended throughout the tube down into the medium along the line of inoculation. At the end of six or seven days, fluorescence was noticeable. This soon extended throughout the tube, the greatest amount being at the surface.

The characteristic of liquefaction is recognized as an important one in separating bacteria. The possession of a proteolytic enzyme sharply separates a strain from those which do not possess this enzyme. The method for determining this

characteristic must be improved, however, in order that comparable results may be secured by workers from the different laboratories. Diagram I indicates the incidence of gelatin liquefaction, among the strains studied.

Growth in nutrient broth

Good growth was secured with all strains when grown in nutrient broth at 37°C. The medium was practically always cloudy, with a pellicle and sediment. Varying degrees of fluorescence were manifested by the cultures. A few were viscid and strung out when a platinum needle was used. This has been mentioned above under the head of Staining Properties. The growth in nutrient broth was not especially characteristic.

Indol formation

A 1 per cent solution of Witte's peptone was used in this test. The tubes were inoculated from a twenty-four-hour broth culture and were incubated at 37°C. for ten days. At the end of this incubation period, 1 cc. of paradimethylamidobenzaldehyde was added to the culture tube. None of the cultures formed indol. With the strains which produced a large amount of fluorescent pigment a red color was very often secured, but this was not due to indol since the culture tubes did not have the characteristic odor of putrefaction. This red color may have been due to the pigment.

Formation of hydrogen sulfide

To study this characteristic, a special medium prepared by Redfield (1912) was used. This was made by adding 300 grams of Witte's peptone and 75 grams of potassium chloride to 700 cc. of boiling tap water. This mixture was heated to dissolve as much peptone as possible, and the solution was then cooled and diluted to 1 liter. It was again boiled, plugged with cotton and allowed to stand in an ice box for at least twenty-four hours. At the end of this time the medium was filtered and transferred to special flasks used in the routine analysis of water.

Mm. liquefaction	0	1-10	11-20	21-30	31-40	41-50	51-60	61-70
No. of cultures	59	0	2	8	15	25	5	4

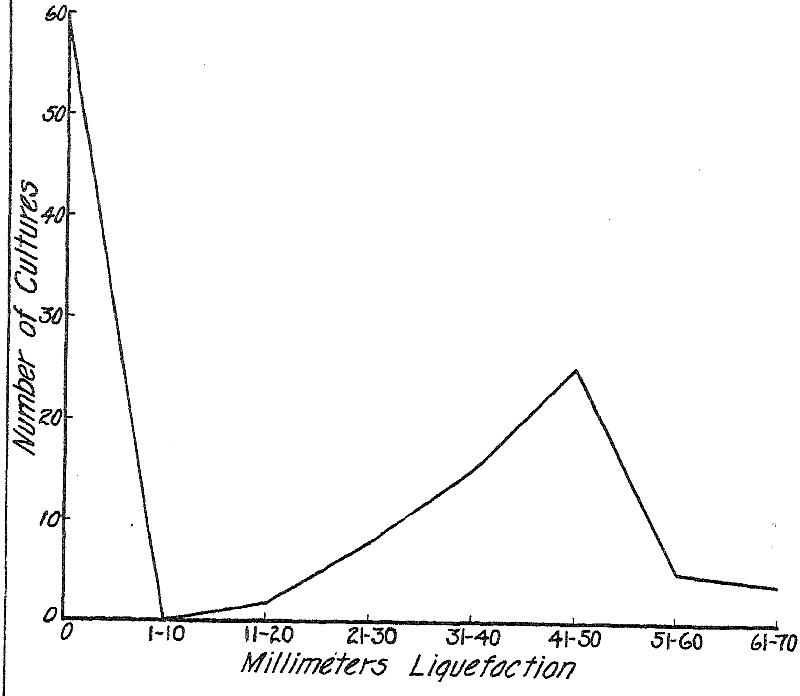

Diagram I. Curve Indicating Incidence of Gelatin Liquefaction by Fluorescent Bacteria

The method here used was a modification of Redfield's, because the flasks used by him in routine water analysis could not readily be adapted to the study of pure cultures. In his method, 10 cc. of his special medium were diluted to 100 cc. in a special flask with 90 cc. of the sample under investigation. This flask was fitted with a ground glass stopper which held the lead acetate paper. In order to adapt this medium to the study of pure cultures, it was made up one-tenth as strong as given above and put into a test tube fitted with a one-hole rubber stopper carrying a piece of glass tubing. A strip of lead acetate paper was held in this tube by means of cotton. Tin foil was twisted about the end of the glass tube. After inoculation from a twenty-four hour culture the tubes were incubated at 37°C. for thirty days. At the end of this period darkening of the paper indicated hydrogen sulfide formation.

Some of the strains studied in this series produced large amounts of hydrogen sulfide. Strains 12, 19, 23, 30, 43, 45, 47, 48, and 52, produced sufficient hydrogen sulfide at 37°C. in much less time than thirty days to darken 2 cm. of a strip of paper 25 by 2 mm. Forty-six of the cultures produced hydrogen sulfide from Redfield's medium.

Redfield has collected the names of the bacteria which have been reported to produce hydrogen sulfide. The following fluorescent bacteria are included.

In two days, *Bacillus fluorescens-non-liquefaciens;* in three days, *Bacillus pyocyaneus;* in ten days, *Bacillus fluorescens-liquefaciens;* in thirty days, *Bacillus cyanogenus* (Fluorescent?); in varying lengths of time, *Bacterium immobile, Pseudomonas fluorescens* (Flügge), *Pseudomonas pilocyanea.*

Edson and Carpenter secured hydrogen sulfide formation in 20 of the 42 cultures which were isolated from maple sap. This gives a percentage of approximately 48, while that obtained with our cultures from water was 46 per cent. The method of Edson and Carpenter consisted in suspending a strip of lead acetate paper above an ordinary broth culture. From these two investigations it would seem that plain broth was about as efficient as Redfield's medium for observing the formation of

hydrogen sulfide by bacteria. In a paper by Chamot and Redfield (1915) on the same subject the statement is made that hydrogen sulfide is more rapidly produced in a mixed culture than in a pure culture. The peptone is probably split to abiuret compounds among which are cysteine and cystine. From these it is easy to imagine how H_2S may be split off. This reaction has been studied by Sasaki and Otsuka (1912), Burger (1914), and Tanner (1917).

Action on carbohydrates

No gas was formed by any of the strains. The reaction in sugar broths was determined after two days' incubation at 37°C. Five cubic centimeters of the media were diluted with 50 cc. of distilled water and titrated, after boiling, with $\frac{N}{20}$ NaOH. Rogers and Davis (1912) commenting on the value of such data for classification work state,

> Mention has already been made of the objections to the use of fermentation of sugars and similar substances. The question of the constancy of these reactions has been the subject of investigation, and while there is some disagreement of opinion among those who have studied the question most carefully it seems that they are at least as constant as any of the characters ordinarily used in classification.

In this study phenolphthalein was used as the indicator, as advised on the 1912 chart of the Society of American Bacteriologists. The ideal method would have been the determination of the true acidity by means of the hydrogen electrode, as has been pointed out by the recent work of Clark and his coworkers.

The rectangular polygons as shown in Diagram II indicate the distribution curves for the various strains with regard to action on glucose, sucrose, lactose and glycerol.

With regard to glucose, the curve shows a single mode which indicates one type of fermentative action. None of the strains formed alkali. The distribution curve for the action on sucrose shows a single mode and one single type of bacteria. All of these bacteria are probably non-sucrose fermenters. A single

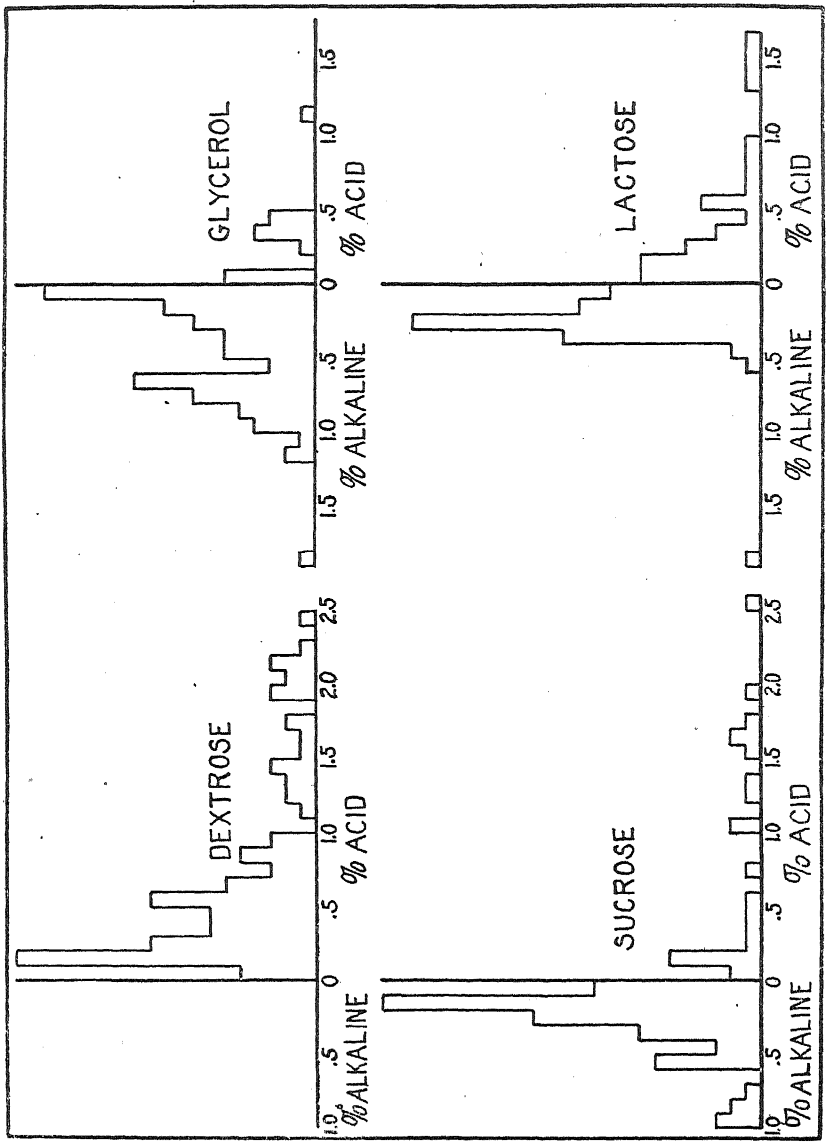

DIAGRAM II. INCIDENCE OF FERMENTATION OF CARBOHYDRATES AND GLYCEROL BY FLUORESCENT BACTERIA

mode is also secured in the distribution curve for lactose. Here, again, a single type of bacteria is indicated. With regard to the action on glycerol, however, two modes are evident which may indicate two types of bacteria. In general, these 100 strains of fluorescent bacteria are alike with regard to fermentation of glucose and non-fermentation of sucrose, lactose and glycerol. Edson and Carpenter (1912) state that with the fluorescent bacteria from maple sap no sharp line of differentiation exists between acid production and no acid production. The distribution curve for the fluorescent bacteria from water indicate the same thing with regard to sucrose, lactose and possibly glycerol.

Growth in Uschinsky's medium

This medium was made up after the following formula and sterilized in the Arnold on three successive days: Distilled water, 1000 cc.; glycerol, 35 grams; sodium chloride, 6 grams; calcium chloride, 0.1 gram; magnesium sulfate, 0.35 gram; dipotassium phosphate, 2 grams.

Vigorous growth was secured in this medium with all strains except no. 10. In many cases the medium was strongly fluorescent. A pellicle was formed by many strains and many of them exhibited a very viscid growth.

Growth in Frankel's medium

This medium was prepared after the following formula: sodium chloride, 5 grams; monocalcium phosphate, 2 grams; ammonium lactate, 6 grams; asparagin, 4 grams; distilled water, 1000 cc.; N NaOH, 20 cc.

The medium was filtered and sterilized in the Arnold sterilizer on three successive days All strains grew abundantly and produced much pigment.

Growth in Sullivan's medium

This medium (Sullivan (1905)) which was found to support good pigment formation was made up as follows: distilled water,

1000 cc.; asparagin, 1 per cent; magnesium sulfate, 0.20 per cent; dipotassium phosphate, 0.10 per cent.

Growth in it was abundant but did not reach a maximum as quickly as in Frankel's medium. None of these strains could reduce the magnesium sulfate to hydrogen sulfide as determined by the method described.

Oxygen relations

All strains were found to be facultative anaerobes. The determination was carried out in a vacuum desiccator in the bottom of which were placed about 50 grams of pyrogallol. The cultures were made on agar slants and placed in the desiccator; and by means of a vacuum pump the pressure was reduced as far as possible under these conditions. After this was done a strong solution of KOH was allowed to be sucked into the bottom of the desiccator by slightly opening the glass stop cock. By this technique it is believed that a minimum supply of oxygen was left.

The desiccator was incubated for five days at room temperature, which was about 25°C. At the end of that time there was visible growth. It was very restricted but quite apparent. Some doubt was felt with regard to the results, since these bacteria have always been described as strict aerobes, but since growth was quite visible, the group number is determined on the ground that they are facultative anaerobes and are able to carry on a limited activity under reduced supplies of oxygen.

Growth in plain milk

This medium was made by thoroughly mixing 100 grams of Merrell-Soule skim milk powder in 1000 cc. of distilled water. It was then beaten with an egg beater, filtered and sterilized in 75 cc. quantities in Erlenmeyer flasks. Inoculations were made from twenty-four hour cultures and the flasks incubated at 37°. The fluorescent bacteria seem to arrange themselves into four general groups with regard to their action on milk.

One group is made up of those cultures which produce prompt

coagulation in two days. Strains 1, 5, 13, 16, 17, 18, 23, 24, 30, 31, 39, 57, and 60, coagulated the milk in this time. The curd formed was solid in consistency and in many cases settled to the bottom of the flask. When peptonization took place this curd was decomposed until the whole flask had assumed a golden color.

Another group produced clearing without coagulation. The rapidity of this action varied. With some of the strains it was evident after twenty-four hours' incubation at 37°. Clearing usually started at the surface—a dark transparent layer being formed which rapidly extended downward. With a few of the strains, however, the entire contents of the flask seemed to change to a thinner consistency. The final product was of a golden color. The green pigment which was often produced in large amounts in this medium, combined with this golden color, to give the solution a striking appearance.

The third type was made up of those strains which seemed to have no effect on the milk in twenty days. It is possible that some change might have been secured, had these cultures been held longer.

The fourth group consisted of those strains which rendered the medium slimy. This was very apparent after twenty days. The strains which possessed capsules always produced a slimy growth in broth and milk.

The Society's chart calls for the determination of the reaction at the end of one, two, four, ten, and twenty days. This was attempted, but erratic results were obtained, since the medium was so highly colored that it was impossible to determine the real end point with phenolphthalein. The green pigment seemed to be the greatest factor in masking the neutral point. Another possible reason why varying results were obtained is that phenolphthalein is not sensitive in solutions which contain ammonia; and much ammonia is formed in the splitting of casein. Were the solutions boiled to remove carbon dioxide, some of the volatile compounds might be driven off and cause a greater error than the carbon dioxide itself. For determining the group number the reaction at the end of the tenth day

was taken. By that time a constant reaction had been established, and continued incubation simply increased it in the same direction.

Growth in litmus milk

This medium was prepared after the same method that was used in the preparation of plain milk. Kahlbaum's azolitmin was used as the indicator. The milk was inoculated from a twenty-four-hour broth culture by means of a platinum loop, and incubated at 37°C. for twenty-five days. A detailed account of all the changes in this medium will not be included. In many cases the tubes turned alkaline at the surface and the blue color gradually extended downward until the whole tube was changed. When peptonization occurred this continued until the whole tube had turned to an orange color.

Many of the strains possessed no proteolytic enzyme and thus produced no change in plain milk. Some of the strains formed alkali which soon gave way to a permanent acid. In many cases the curdling of the milk could not be entirely explained by the concentration of the acid. It was doubtless due to the presence of a rennin-like enzyme.

Peptonization of casein

With most of the strains casein peptonization was apparent in the flasks used in the titration of the acidity of milk, but with the others it was impossible to tell whether the casein was attacked. In order to determine this character, Hasting's (1904) milk agar was used. This was prepared by cooling a tube of melted agar and adding to it 1 cc. of sterile skimmed milk. This was then poured into a sterile Petri dish and thoroughly stirred. When solidified, it was inoculated by making streaks on the surface, after which the plates were incubated for five days at 37°C. A clear zone appeared around the line of inoculation on those plates which were inoculated with the strains capable of peptonizing the casein. To verify this conclusion, dilute acetic acid was added to the plate, and if the zone remained clear the strain was recorded as one which would peptonize.

Forty-two strains were able to decompose casein. This reaction, of course, was more marked with a few of the forms than with the majority.

Temperature relations

The temperature relations of any group of bacteria are very important. It is often believed that since an organism is found in nature outside the body, it has its optimum temperature and grows best at temperatures near 20°C., and that growth is restricted at 37°. The literature on fluorescent bacteria indicates that very few of these forms refuse to grow at 37°. All the cultures used in this study were found to grow well at this temperature. The amount of change brought about by bacteria may be said, generally speaking, to be a function of the incubation temperature and period of incubation, in accordance with the laws which govern enzyme action and the relation of temperature thereto.

Fluorescence

This characteristic was a necessary requisite for the inclusion of a culture in the investigation. It is closely related to the pigment which these bacteria form. Some bacteriologists have attempted to separate *B. pyocyaneus* and *B. fluorescens-liquefaciens* on the ground that *B. pyocyaneus* possessed no fluorescent pigment. This separation is probably more apparent than real. There were all gradations among the cultures of this series with regard to this characteristic. The forms which liquefied gelatin and casein produced more fluorescent pigment than those which did not break up these two compounds.

Color in nature

The theories with regard to color or pigment formation are reviewed by Sullivan (1905). He divides pigments into two divisions, viz: structural and pigmental. Pigments in nature are divided into: (a) Pigments of direct importance, as in respiration; (b) derivatives of such pigments; (c) waste products,

or derivatives of such; (d) introduced pigments; (e) reserve pigments or pigments associated with reserves.

Sullivan also reports Beyerinck's (1891) division of chromogenic bacteria, which is as follows: (a) Chromophorous bacteria—forms in which the pigment serves some purpose in the cell, as chlorophyll; (b) chromoparous bacteria—forms which excrete the pigment as a waste substance; and (c) parachrome bacteria—forms which retain the pigment in their cells.

The subject of bacterial pigments has been much discussed; but only a few of the publications will be mentioned here. Wasserzug (1887) studied *B. pyocyaneus* or the organism of green pus and found that in the same cultures not all the cells produced the same pigment. To avoid this variation as far as possible, he worked with cultures which had been rejuvenated by successive transfers and inoculations into rabbits. Wasserzug states

> Il semble qu'on puisse distinguer deux periodes dans la vie de l'organisme coloré: dans la premiere il prefers et accommoda a ses besoins son milieu de culture; dans la second, il produit et secrete, la matière colorante.

He tried the effects of antiseptics and found that the points where pigment formation and growth stopped differed but slightly. This would seem to indicate that the colored pigment was merely a waste produce resulting from cellular metabolism.

Jordan (1899) studied the conditions under which the diffusible fluorescent pigment was produced. He used synthetic media and concluded that the presence of phosphorus and sulfur was essential to vigorous fluorescing properties. He added that the fluorescent property might be of no benefit to the cell.

Babes (1889) studied the colored materials from *B. pyocyaneus*. To secure his pigments he inoculated neutral peptone gelatin with a strain isolated from an abscess. The medium was inoculated and left for six weeks at room temperature. The color varied and an odor of linden flowers was noticeable. Babes used solvents and distillation methods to remove his pigment,

and probably secured "the pyocyanin" of Fordos (1863). This was blue in alkaline and red in acid solution, and crystallized in rhombic crystals. A red-brown substance was also secured which was greenish in refracted light, soluble in water and insoluble in chloroform. In acid solution fluorescence was lost, but was acquired again in alkaline solution. By distillation a colorless substance with a peculiar odor was obtained. This differed from the original and was supposed to be a decomposition product.

Thumm (1905) found that all species produced the same pigment, which differs from the reports of other investigators. He was unable to confirm the findings of others that several pigments were produced. He found that this group was made up of vigorous alkali formers, and that glucose was fermented to acids which were neutralized later by the formation of ammonia.

Boland (1899) believes that two pigments are formed, a fluorescent one, which is formed by many other bacteria, and pyocyanin which changes into a red brown pigment by oxidation.

Krause (1900) studied the symbiosis of *B. pyocyaneus* with pus formers and found that the aromatic odor was almost always present, and that as long as *B. pyocyaneus* predominated over the pus formers, the green color did not appear. When *B. pyocyaneus* cells were removed from the culture, they again formed the green pigment. Certain gases were tried, and with hydrogen good growth was secured but no pigment. With CO_2 and the vacuum method no growth was secured. In his study of the pigments the following were secured:

(1) Water extract, greenish yellow fluorescence; (2) 80 per cent alcohol, yellowish green fluorescence; (3) glycerol, blue-green fluorescence; (4) Amyl alcohol, grass green fluorescence; (5) chloroform, blue; (6) ether, yellow blue.

Nogier, Dufourt and Dujol (1913) studied a strain from a lesion and found a red pigment of the color of vinegar along with the fluorescent and red-brown pigment usually described. This red pigment was formed in glycerol broth on the fourth day. Acid and alkali seemed to suppress pigmentation, as did pure oxygen.

Gessard (1890) believed that two pigments were formed, a fluorescent green and pyocyanin.

Sullivan (1905) finds that pyocyanin formation is independent of the presence of phosphate or sulfate. He did find, however, that phosphorus and sulfur were essential. He recognizes a fluorescent pigment and pyocyanin and states that the same variety of *B. pyocyaneus* may be made to display either of these functions or both. Sullivan believes that "the production of pigment is not an essential vital act. As it is of no discoverable advantage to the organism possessing the power of producing it, its production is purely accidental."

In a study of this pigment by the writer, a 4 liter flask of plain broth was inoculated with strain 37 and left for eight weeks at a temperature of about 25°C. At the end of that time the medium had assumed a dirty green color with a heavy precipitate in the bottom of the flask. When the flask of broth was tested for growth, a large number of living bacteria were found. This culture was filtered through paper into a large bottle from which different portions were taken for study.

About $1\frac{1}{2}$ liters were precipitated with lead acetate and allowed to stand over night. In the morning this material was filtered and divided into three portions. Each of these was extracted with ether, chloroform and ligroin. The chloroform was the only solvent which removed any of the pigment. It removed a blue pigment which was increased in amount by subsequent shakings in a separatory funnel. In four or five days this blue chloroform solution changed to a red when left in bright light. No reagents seemed able to change it back to the green color. In the dark the blue chloroform solution changed from a deep blue color to a dark green which was permanent.

Some of the chloroform solution was allowed to evaporate in a crystallizing dish. A black residue which possessed crystalline structure was left and this had a very strong odor similar to some of the aromatic ammonium bases. This substance was soluble in alcohol and water, insoluble in ether and red in acid solution. Neutralization restored the green color again.

The work here reported agrees with that reported by the

early authors. Ledderhose (1888) believes that this pigment which has been called pyocyanin belongs to a group of aromatic substances closely related to the anthracene group. It was thought that this pigment might be related to anthocyanin which is supposed to have some relation to the flavon or xanthon groups. This may exist in the form of a colorless glucoside in the plant and be capable of oxidation only after it has been liberated. Repeated tests by Molisch's reaction failed to demonstrate its glucosidal character either before or after hydrolysis. More work on this subject is planned.

Reduction of nitrates

Nitrate reduction tests were made on media containing distilled water, 1000 cc.; Witte's peptone, 1 gram; and potassium nitrate, 2 grams.

Thirty cubic centimeters of this medium were sterilized in small Erlenmeyer flasks. These were inoculated from a twenty-four-hour broth culture and incubated for five days at 37°C. At the end of this period 1 cc. of the nitrate broth culture was removed by a sterile pipette and diluted to 50 cc. in a Nessler tube with nitrite free water.

The method used for determining nitrites was that usually employed in water laboratories, and it is believed that it is not too delicate for bacterial work if blank determinations are made. To each of the Nessler tubes prepared above was added 1 cc. of an acid solution of naphthylamine hydrochloride and 1 cc. of a saturated solution of sulfanilic acid. The tubes were allowed to stand for thirty minutes in the colorimeter before being examined. Control tests were always made at the same time.

Fifty-one of the strains reduced potassium nitrate when incubated for five days at 37°C.

Formation of ammonia

The nitrate broth cultures were used for this purpose. At the end of ten days 2 cc. of this broth culture were withdrawn and diluted to 50 cc. with ammonia free water. Nessler's

reagent was added to the tubes and control tubes, and comparison made in a colorimeter used for determining ammonia in water analysis.

Fifty-two of the 100 cultures produced ammonia in ten days at 37°C. Plain nutrient broth cultures were often tested for ammonia production, and all strains were found to produce ammonia. The plain broth cultures were alkaline to phenolphthalein.

Vitality of fluorescent bacteria

The members of this group seem to be able to resist very unfavorable conditions. No special experiments have been made, but much evidence from a number of sources is available.

Broth suspensions of *B. pyocyaneus* were suspended in the Urbana septic tank and daily counts made. The number of bacteria increased regularly until the end of the period, which indicates that this bacterium is able to live in such an environment.

It has been noticed that tubes of supposedly sterile culture media which spoiled were often infected with bacteria of this group. Thus it is apparent that these bacteria are able to resist high temperatures for short periods. It is well known among physicians that once a hospital is infected with *B. pyocyaneus*, it is disinfected with great difficulty.

Cultures of these bacteria live for a long time on laboratory media. Agar slants which have been allowed to dry for six months at room temperature were found to support living fluorescent bacteria. Cultures of strains 22 and 37 were left for a year and a half with infrequent transfers and in each case good growth was secured from the old cultures. These cultures did however lose some of their pigment-producing property.

Rettger and Sherrick (1911) report an example of resistance by a member of this group. They state that an old culture of *B. pyocyaneus* began to lose its property of producing the green pigment. An agar slant of this bacterium was placed on the top of an incubator in April and left until October. When examined at this time the agar was dried to a hard mass and it was thought unlikely that any living bacteria were present.

Transfer to fresh media, however, gave good growth and much pigment was produced.

Burge and Neill (1915), in a paper on the comparative resistance of fluorescent and non-fluorescent bacteria to ultra-violet light, conclude that fluorescent bacteria are better able to resist ultra-violet light than those which do not produce this pigment. Their cultures of fluorescent bacteria were secured from among those which form the basis of this investigation. Table 3 indicates the results which they secured with the fluorescent bacteria. The numbers in parentheses are those which the cultures bear in this paper; the other numbers are those which Burge and Neill assigned to them.

TABLE 3

Germicidal action of ultra violet light on fluorescent bacteria

TIME OF EXPOSURE	NUMBERS OF BACTERIA PER CUBIC CENTIMETER						
	1 (2)	2 (3)	3 (13)	4 (15)	5 (22)	6 (25)	7 (37)
seconds							
0	72 M*	80 M	28 M	70 M	75 M	33 M	25 M
20							
40							
60	750	813	55	210	460	12	88
80	459	623	29	79	211	10	20
100	223	221	25	56	113	7	14
120	128	102	20	29	103	5	12
140	97	88	15	18	56	4	8
160	81	73	12	13	38	3	2
180	43	29	10	6	25	3	1
200	31	15	3	3	11	2	1

* Million.

This table indicates that these bacteria have a high resistance to ultra-violet light. In order to secure some basis for comparing this character, the same authors exposed such common forms as *B. coli* (*communis*), *Pseudomonas violacea*, *Sarcina lutea* and *B.* (*proteus*) *vulgaris*, which are not fluorescent, to the effects of the ultra-violet light. In no case were any of these bacteria able to resist the effects of the ultra-violet light for more than 200 seconds. Most of them could not survive an exposure of

160 seconds. With the fluorescent bacteria, however, the results are quite different. In each case a few cells were found alive at the end of 200 seconds. Curves accompany the paper by these investigators which indicate that the death rate follows the monomolecular law. Burge and Neill explain this resistance of fluorescent bacteria to ultra-violet light by assuming that "the fluorescent bacteria protect themselves from the coagulating effect of the ultra-violet light by converting the short wave lengths to longer waves and hence disposing of energy of the absorbed short waves." Non-fluorescent bacteria were unable to do this.

Divisions of strains into groups

The group numbers allow the following separation of strains.

2	121.2332133	61, 45
1	121.2333133	47
1	122.2333133	70
2	221.2222132	22, 15
1	221.2222133	13
2	221.2223132	23, 20
6	221.2223133	24, 17, 2, 50, 31, 3
4	221.2232133	34, 6, 88, 86
4	221.2233133	71, 29, 26, 25
1	221.2322132	27
1	221.2323132	30
1	221.2323133	48
2	221.2332132	62, 59
14	221.2332133	99, 91, 94, 84, 85, 73, 46, 28, 21, 16, 87, 14, 10, 74
2	221.2333132	69, 66
12	221.2333133	98, 90, 75, 72, 67, 64, 63, 37, 35, 32, 12, 65
1	222.2222133	19
1	222.2223132	60
6	222.2223133	93, 83, 80, 79, 78, 33
1	222.2232133	95
1	222.2233132	82
6	222.2233133	92, 68, 81, 52, 96, 89
1	222.2322132	5
1	222.2323133	100
3	222.2333132	39, 57, 55
10	222.2333133	97 77, 76, 56, 54, 53, 43, 9, 8, 7
13	222.2332133	58, 51, 49, 44, 42, 41, 40, 38, 36, 18, 11, 4, 1

Twenty-seven groups are thus secured, many of which are separated by but one characteristic from those closely related to them. With the group number as it now stands, it is possible to obtain 276,480 different types of bacteria.

Diagram III presents a brief characterization of the action of these cultures on some of the more important substances. Such a diagram was first used by Rogers and Clark (1912).

Neglecting nitrate reduction and gelatin liquefaction as variable characters of little value for classification, the following nine groups are obtained:

			Strains
22±	. 233±	133	49
22±	. 223±	133	15
22±	. 222±	133	14
22±	. 233±	132	7
22±	. 222±	132	5
12±	. 233±	133	4
22±	. 232±	132	3
22±	. 223±	132	1
22±	. 232±	133	2

Inspection of the above numbers indicates a very close relationship. Barring the four spore formers, there is an apparent intergrading of characters. Were all of the strains strictly considered as non-fermenters of lactose and sucrose, as might be inferred from Diagram II, the number of groups would be further reduced to five. They might be regarded as falling into two groups with regard to glycerol, and this characteristic might be regarded as a basis for separation.

DISCUSSION OF RESULTS

A comparative study of 100 strains of fluorescent bacteria from water by means of the group number system as expressed on the descriptive chart of the Society of American Bacteriologists, 1912, places them in 27 groups. Twelve of these are made up of but one strain and are separated from the adjacent groups by but one characteristic. If gelatin liquefaction and nitrate reduction are excluded as variable characteristics for classification purposes, as has been suggested by different investigators,

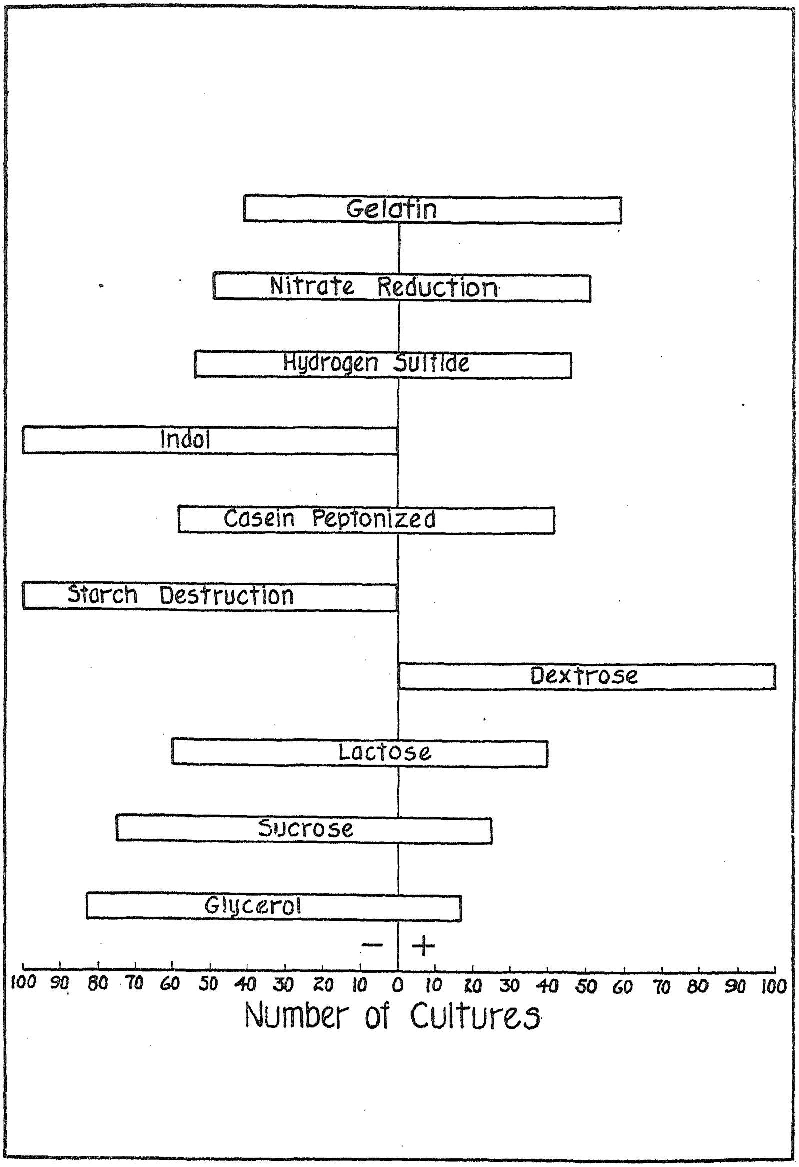

Diagram III. Action of Fluorescent Bacteria on Various Substances

the 100 strains fall into 9 groups all of which are very closely related.

The group number probably does not separate bacteria along natural lines, but it does constitute a convenient method for considering the characters of such closely related bacteria as make up the fluorescent group. It obviates the necessity for bacterial names which have very little meaning in present bacterial work. Especially true is this in regard to the fluorescent group. The group number places equal emphasis on all the determinations which it expresses. It is quite probable, too, that many of the inadequacies of the group number may be explained by the lack of proper methods for determining the characters for which it calls.

Four of the cultures form endospores, which have been accepted by bacteriologists as a reliable and important basis for separating bacteria. De Bary placed so much importance on this that he described minutely the formation and germination of spores. This characteristic is one of importance which indicates that the fluorescent bacteria may not be a genetic group. Edson and Carpenter, however, report no spore-forming fluorescent bacteria in their study of maple sap.

The presence of a proteolytic enzyme for gelatin might be of more value in classification were a satisfactory method available for detemining its presence. With the fluorescent bacteria this has been taken as the sole difference between certain members of the group. The 100 strains forming the basis of this study were about evenly divided with respect to this characteristic.

Gelatin liquefaction paralleled casein digestion closely, although sixteen strains which liquefied gelatin failed to split casein. Of the cultures which liquefied gelatin, the majority liquefied between 40 and 50 mm. The others graded away from this group and one may infer that the determination, as it is now made, is not sufficiently delicate for classification purposes. Those strains which required four or five months for liquefaction to appear might be regarded as the links between the liquefiers and the non-liquefiers, or they might represent transitional forms which are in the act of acquiring or losing this characteristic.

Investigations with regard to better technique for determining gelatin liquefaction are under investigation in this laboratory.

The strains are all reported as facultative anaerobes. This is another determination called for in the group number for which there is no satisfactory technique. Formerly much importance was attributed to growth along the line of inoculation in stab cultures. This is beset with too many objections. A medium itself may contain sufficient dissolved oxygen to support growth. Evidence of growth in the closed arm of the fermentation tube has also been used to determine anaerobiosis. This limits the determination to the particular substance from which the oxygen was taken. A standard technique for this determination would have much significance.

With regard to diastatic action on starch, the personal equation is given too much importance in recording this characteristic on the Society's chart.

All of these cultures correlate with regard to fermentation of glucose, production of fluorescent pigment, absence of diastasic action on starch, negative indol formation, and probably non-fermentation of sucrose and lactose. The modes in the curve of Diagram II indicate that there is probably one type of action on lactose and sucrose and possibly two types with regard to glycerol. However, with glycerol one of these modes is quite near the other and close to the line where experimental error might connect it more evidently with the other. Since these characters agree for so many cultures, all of which are from widely separated sources, they are probably of significance. The absence of diastatic action presupposes no amylase and correlates well with the action on the other carbohydrates, lactose and sucrose. As stated before, it is recognized that the use of phenolphthalein as the indicator in determining reactions is arbitrary, and that determination of true acidity by means of the hydrogen electrode may prove that all of these strains were unable to ferment carbohydrates. Negative indol formation may indicate that peptone is not split to amino acids and other products including tryptophan which is the precursor of indol.

The explanation of the correlation between acid formation in

glucose broth and production of fluorescent pigment is probably bound up with the structure of pyocyanin and its formation by the bacterial cell. If the opinion of Ledderhose is accepted that this pigment is a derivative of the anthracene group, it is possible that no apparent relation exists. The fluorescent bacteria produce a green diffusible pigment along with which there is a large amount of ammonia. The pigment itself is probably basic in character.

SUMMARY

1. Of a series of 100 strains of fluorescent bacteria, isolated from water, all cultures correlate with regard to the production of fluorescent pigment, (produced very profusely when the bacteria are grown in Frankel's solution), formation of acid in glucose broth, absence of diastasic action upon potato starch, negative indol formation and non-fermentation of sucrose and lactose.

2. These cultures, when studied according to the group number as expressed on the descriptive chart of the Society of American Bacteriologists, fall into 27 groups.

3. The fluorescent bacteria are about evenly divided with regard to gelatin liquefaction. The test is not delicate when applied to this group and requires further study.

4. Four of the 100 strains are spore formers. This characteristic is recognized as one which logically separates a bacterial species.

5. With the exception of the four spore formers, the fluorescent bacteria in water, as indicated by this study, constitute a homogeneous group of bacteria, difference appearing only in regard to gelatin liquefaction, formation of hydrogen sulfide and perhaps fermentation of glycerol.

It is a pleasure to acknowledge my indebtedness to Prof. Edward Bartow and Prof. H. A. Harding for their personal interest and valuable suggestions throughout this work.

REFERENCES

BABES 1889 Note sur quelques matières colorantes et aromatiques produites par le bacille pyocyanique. Compt. Rend. Soc. Biol., **41**, 438–439.

BELLI 1902 Chemische microscopische und bakteriologische Untersuchungen ueber den Hagel. Cent. f. Bakt., II Abt., **8**, 445–446.

BEYERINCK 1891 Die Lebensgeschichte einer Pigment Bakteria. Bot. Zeit., **49**, 705.

BOLAND 1899 Ueber pyocyanin den blauen Farbstoff des *Bacillus pyocyaneus*. Centralbl. f. Bakt., **25**, 897–902.

BUCHANAN 1915 Nomenclature of the Coccaceae. Jour. Inf. Dis., **17**, 528–541.

BURGE AND NEILL 1915 The comparative rate at which fluorescent and non-fluorescent bacteria are killed on exposure to ultraviolet light. Am. Jour. Physiol., **38**, 399–403.

BURGER 1914 Formation of Hydrogen Sulfid from Cystin by Bacteria. Arch. f. Hyg., **82**, 201–211.

CHAMOT AND REDFIELD 1915 Studies on culture media employed in bacteriological examination of water. Jour. Am. Chem. Soc., **37**, 1606–1630.

COMMITTEE 1898 Procedures recommended for the study of bacteria with special reference to greater uniformity in the description and differentiation of species. Being the report of a committee of bacteriologists to the committee on purification of water supplies of the American Public Health Association. Am. Pub. Health Assoc. Proc., **23**, 60–100, 1898.

CONN, H. W. 1906 Classification of Dairy Bacteria. Ann. Rep. Conn. Agr. Exp. Station, 91–203.

CONN, H. W. 1910 Practical Dairy Bacteriology. Orange Judd & Co., 217.

CONN, H. J. 1915 A study of *B. subtilis* by the classification card. Science, N. S., **41**, 618.

CORNWALL 1915 Classification of bacteria for the purpose of water analysis. Indian. Jour. Med. Res., **2**, 352–368. Original not seen. Reviewed in Chem. Abstracts, **9**, 328.

EDSON AND CARPENTER 1912 The green fluorescent bacteria occurring in maple sap. Vermont Agr. Exp. Station. Bull. No. 167, 521–599.

EISENBERG 1914 Untersuchungen über die Variabilität der Bakterien. Centralbl. f. Bakt. I Abt., **73**, 466–470.

FORDOS 1863 Recherches sur la matière colorante des suppurations bleues-pyocyanine et pyoxanthose. Compt. Rend., **56**, 1128–1131.

FULLER AND JOHNSON 1899 On the differentiation and classification of water bacteria. Jour. Exp. Med., **4**, 609–626.

GAGE AND PHELPS 1902 On the classification and identification of bacteria with a description of the chart system in use at the Lawrence Experiment Station for records of species. Am. Pub. Health Assoc. Proc., **28**, 494–505.

GESSARD 1890 Des races du bacille pyocyanique. Ann. de l'Inst. Past., **5**, 65.

GRIFFON 1909 Sur le rôle des bacilles fluorescent de Flügge en pathologie vegetale. Compt. Rend. Acad. Sci., **149**, 51–53.

HARDING 1910 The constancy of certain physiological characters in the classification of bacteria. N. Y. (Geneva) Agr. Exp. Station, Tech. Bull. No. 13.

HARDING, H. A. AND PRUCHA, M. J. 1908 The bacterial flora of Cheddar cheese. N. Y. (Geneva) Agr. Exp. Station, Tech. Bull. No. 8.

HARDING, MORSE AND JONES 1909 The bacterial soft rot of certain vegetables. N. Y. (Geneva) Agr. Exp. Station, Tech. Bull. No. 11.

HARRISON 1898 Bacterial content of hail stones. Bot. Gaz., **26**, 211–214.

HASTINGS 1904 The action of various classes of bacteria on casein as shown by milk agar plates. Centralbl. f. Bakt., II Abt., **12**, 590–593.

JOHNSTON, W. 1894 On the grouping of water bacteria. Am. Pub. Health Assoc. Proc., **20**, 445–449.

JORDAN 1899 The production of fluorescent pigment by bacteria. Bot. Gaz., **27**, 19–36.

JORDAN, E. O. 1903 The kinds of bacteria found in river water. Jour. Hyg., **3**, 1–27.

KRAUSE 1900 Beiträge zur Kenntnis des *Bacillus pyocyaneus*. Centralbl. f. Bakt., I Abt., **27**, 769–775.

KRUSE 1896 The fluorescent group of bacilli. Die Mikroorganismen. Flugge 3rd edition, Vol. II, 289.

LAUBACH 1916 Studies on aerobic spore-bearing non-pathogenic bacteria: Spore-bearing organisms in water. Jour. Bact., **1**, 505–506.

LEDDERHOSE 1888 Ueber den blauen Eiter. Deut. Ztschr. f. Chir., **28**, 201.

MUIR AND RICHIE 1913 Manual of Bacteriology, p. 110.

NIEDERKORN 1900 Vergleichende Untersuchungen über die verschiedenen Varietäten des *Bacillus pyocyaneus* und des *Bacillus fluorescens liquefaciens*. Centralbl. f. Bakt., I Abt., **27**, 749–750.

NOGIER, DUFORT AND DUJOL 1913 Contribution a l'etude des pigments du *bacillus pyocyaneus*. Jour. Physiol. path. gen., **15**, 633–635.

RAHN AND HARDING 1914 Die Bemühungen zur einheitlichen Beschreibung der Bakterien in Amerika. Centralbl. f. Bakt., II Abt., **42**, 385–393.

REDFIELD 1912 A study of hydrogen sulfid production by bacteria and its significance in the sanitary examination of water. Thesis. Cornell University.

RETTGER AND SHERRICK 1911 Studies on bacterial variation. Jour. Med. Res., **19**, (N. S.) 265–284.

ROGERS AND DAVIS 1912 Methods of classifying the lactic acid bacteria. U. S. Dept. Agr., Bur. An. Ind. Bull. No. 154.

RUZIKA 1898 *Bacillus pyocyaneus* and Bac. *fluorescens liquefaciens*. Arch. f. Hyg., **34**, 140. Experimentelle Studien ueber die Variabilitat wichtigen Charaktere des *B. pyocyaneus* und *B. fluorescens liquefaciens*. Centralbl. f. Bakt., **24**, 11–17.

SASAKI AND OTSUKA 1912 Experimentelle Untersuchungen über die Schwefelwasserstoffentwicklung der Bakterien aus Cystin und sonstigen Schwefelbindungen. Biochem. Ztschr., **39**, 208–215.

SCHMELCK 1888 Steigerung des Bakteriengehaltes im Wasser während des Schneeschmelzens. Centralbl. f. Bakt., **4**, 195–199.

SULLIVAN 1905 Synthetic culture media and the biochemistry of bacterial pigments. Jour. Med. Res., **14**, 109–160.

TANNER 1917 Studies on bacterial metabolism of sulfur. Jour. Bact., **1**, 585–593.

THONI 1911 Biologische Studien über Limonaden. Centralbl. f. Bakt., II Abt., **29**, 616–643.

THUMM 1905 Beiträge sur Biologie der fluoreschirenden Bakterien. Arb. a. d. Bakt. Inst. der Hochschule. **1**, 291–377. 1895 Original not seen. Reviewed by Smith. Bacteria in Relation to Plant Diseases. Vol. 1, 238.

WARD, M. 1897 Fifth report to the Royal Society Water Research Committee. Proc. Roy. Soc. London, **61**, 415–423.

WASSERZUG 1887 Sur la formation de la matière colorante chez le Bacillus pyocyaneus. Ann de l'Inst. Pasteur., **1**, 581–591.

WINSLOW 1914 The characterization and classification of bacterial types. Science, N. S., **39**, 77–90.

THE SCIENCE OF BACTERIOLOGY AND ITS RELATION TO OTHER SCIENCES[1]

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Bacteriology is a child of many adoptions, ever precocious, but not yet fully mature. Born with a definite mission to serve and to save, it has re-created pathology, given inspiration and new life to botany and zoology, contributed generously of its substance to agriculture and home economics, and lent itself as the framework around which modern hygiene and preventive medicine have been built. Yet all the while it has conducted itself in competent hands as a pure science.

Bacteriology has, however, been the victim of gross paternalism by those sciences which it has come to redeem. Botany in particular has long laid a claim to it; but it is to pathology that it has been holding itself in bondage, as the result perhaps of their close affiliation since the early days of Pasteur, Lister and Koch. Like the sturdy youth who has long passed his majority, but who suddenly realizes his own powers to conduct affairs for himself or to claim due recompense for his arduous labors in support of a stern father or guardian, bacteriology must and will emerge from its servile state.

Nor has the bacteriologist himself been entirely free from blame. In spite of abundant opportunities for the most valuable scientific researches, and for the promulgation and application of important principles and relationships, faulty and incomplete preparation have too often taken the place of long and thorough training in the subject. Many positions have been, and are, even

[1] Address of the President of the Society of American Bacteriologists, delivered at Nineteenth Annual Meeting, Washington, December 28, 1917.

today, filled by persons whose preparation has been sadly inadequate. How often the newly appointed bacteriologist to a municipal or state board of health is a physician whose complete knowledge of bacteriology has been acquired in the short period of eight or ten weeks of a regular medical school curriculum!

The bacteriologist can not be made in a day. Dr. Moore in his admirable presidential address dwelt upon the need of thorough training in bacteriology. No wonder that he was dismayed at the failure to evolve a finished product at the end of the regular college course. How can a bacteriologist be made in three or six months, when it takes at least as many years to produce a chemist, a physicist or an electrical engineer? It is my firm conviction that we cause irreparable injury to our science when we recommend for positions men or women who have not had the same advanced training that is as a rule required of the chemist, for example. They must be able to do more than to pour gelatin and agar plates and to count colonies.

The trained bacteriologist of the future will need a deep and broad foundation upon which he is to erect his superstructure. A knowledge of general biology and chemistry will be as essential for him as arithmetic and algebra are for the man who is entering upon a course in higher mathematics. To this must be added elementary physics and a reading knowledge of French and German, at least.

It is indeed pathetic to see young men or women of undoubted ability and promise apply for admission into the graduate department of a university and to see them denied admission because of inadequate preliminary training. Pathetic, I say, because of the sudden realization that comes over such earnest seekers for the truth, a realization that they are unfit in spite of their natural ability.

With a general college training covering the above subjects, the student who has chosen bacteriology as his chief field of activity and life work has a wide choice of closely allied subjects, as for example biochemistry, protozoology, parasitology, pathology, sanitary chemical analysis, sanitary engineering, and hygiene and public health. He must at no time lose sight of

the disciplinary value of this or that subject, but his main interest should be in those channels which will aid him materially in his mastery of bacteriology as a real science, and in its many important relationships.

Bacteriology differs from all other sciences. Its technique and methods of experimentation and control are its own. It has its own problems. Yet like other sciences, and even to a greater degree, it must borrow from, and give to, other fields of study. Its relations and applications to the various industries, to hygiene, medicine, etc., are many; but at the very foundation its principles are as thoroughly scientific as those of any other branch of knowledge. Coulter has said that what determines a subject as a real science is not only the subject matter itself but also the purpose with which it is pursued.

To study bacteria as the ornithologist does birds, or the geologist, rock formation—in other words, to learn nature's secret in so far as it is revealed by this large group of living organisms—is indeed as truly a scientific inquiry as the most profound investigation into the structure of the protein molecule. Nor need such a study be limited to organisms which are of no interest to the student of medicine. The general student of bacteriology is just as much entitled to a knowledge of the typhoid bacillus in its relation to environment and natural habitat as he is to a full understanding of the characters of *Bacillus subtilis*, what its common places of residence are, and what its economic rôle may be.

Bacteriology has too wide a range of activity and influence to confine it to the usual Medical School curriculum; and like physics, geology and biochemistry, it requires an academic or university atmosphere. To those who are in quest of scientific pursuits it presents many problems of profound interest, as for example those of biological classification, variation, cell growth and metabolism, the response of microorganisms to stimuli, enzyme action, organic synthesis and decay, etc. In fact the very problems of life's origin and of death belong as much to the realm of bacteriology as to any other science. There is no necessity of dwelling here on its many practical applications, which need not be any the less scientific.

If there is any one related science which, to the bacteriologist, is more important than any other, it is chemistry. It is indeed unfortunate that there are few chemists who are also bacteriologists. It is to be regretted also that comparatively few bacteriologists are familiar with the principles of chemistry. The latter assertion is well borne out by the failure of systematists to evolve even moderately successful classification schemes, and by the action of committees on standard methods whose choice of standard methods has not always been the wisest. It required a Jensen to show the way to a newer and more rational system of bacterial classification; and it was not until physical chemists came to our aid that a scientific and practical method of determining the H ion concentration of a culture medium was supplied. The study of antiseptics and chemical disinfectants has received a new impetus through the work of Chick and Martin (1908) and the recent researches of Dakin (1916) and his associates.

Pasteur's victory over Liebig in their noted controversy on the exact relationship of the yeast cell to fermentation was a victory for the biologist. Buchner's researches on the enzymes of yeast somewhat reversed the tables again, and today we are more and more being led to believe that practically all transformation of bacterial substrates is brought about through the immediate agency of an enzyme, whatever that may be.

Exhaustive chemical investigations into the composition of bacteria and their multitudinous products are necessary before we can acquire an understanding as to what bacteria in reality are and what they can do. The investigations of Von Nencki and his pupils promised a wide development of this field. Others have made most valuable contributions, as for example Brieger, Kühne, and the Martins. In more recent times, the researches of Tamura on the chemistry of bacteria, are of particular interest; also the work of Armand-Delille (1913) and his associates on the significance of amino acids and di-amines in culture media, and the investigations of others (Sasaki, (1912) Otsuka (1916), etc.) on the possible rôle of polypeptides in bacterial nutrition.

Bacterial nutrition is essentially a biochemical problem, but should make an emphatic appeal to the bacteriologist. It has as yet been but little explored. The impelling need for appropriate synthetic media in the biometric or quantitative study of physiological activities of bacteria remains today practically unsupplied. We are as yet in the dark regarding the real food requirements of bacteria.

Until very recently it was a common assumption that practically every sort of organic matter is food for bacteria. Today we are made to realize that the problem of dietetics in bacteriology is as real as in animal physiology. Indeed we are observing more and more that the physiology of bacteria and of animal organs is not so very unlike. The processes involved in the pancreatic digestion of protein are in a large measure reproduced by the proteolytic enzymes of organisms of the *B. subtilis* and *Proteus vulgaris* types, certainly by the organisms of putrefaction. Bacterial enzymes are able to bring about the rapid cleavage of true proteins with the production of essentially the same decomposition products as those which are formed in ordinary tryptic digestion, namely albumoses, peptones and polypeptides, various amino acids (leucine, tyrosine, etc.) and tryptophane. Certain products are, of course, characteristic of the bacterial cell, as for instance indol, the aromatic oxy-acids, and the ultimate decomposition products, ammonia, hydrogen, methane, carbon dioxide, etc.

The problems of assimilation of food are essentially the same for the bacterial and animal cell. Foods must be of very simple composition in order that they may be utilized for cell growth. This was demonstrated but a few years ago by Loewi, Abderhalden and others in animal physiology. In the words of Abderhalden, "It is as essential to break down complex nitrogenous food substances into their simple components, before they can be utilized, as it is to reduce the walls of an old church brick by brick before they can be made over into a modern schoolhouse." Recent investigations in our laboratory have impressed most vividly upon us the same fact with reference to bacterial nutrition.

We have been led through oft-repeated experiments on animal and vegetable proteins to conclude that all bacteria are unable to derive nourishment from native proteins, and that in a medium in which there is no other possible source of nitrogen than the proteins themselves they will thrive no better than in a chemically pure saline solution. When cleavage-producing agents, like a proteolytic enzyme, are present, the complex protein molecules are broken up and, at least in part, made available for cell nutrition.

We have been able to show also that albumoses and the more complex peptone fractions of Witte's and other commercial peptones are like stone to the bacterial cell. Not only are bacteria unable to utilize these substances without preliminary cleavage or separation into their constituent parts, but many organisms are without the ability to produce the enzymes necessary to prepare these complex organic substances for cell nutrition. The Coli-typhi-dysenteriae group furnishes notable examples of such organisms. With but few exceptions there appears to be a correlation between this property and that of gelatin liquefaction.

The question may well be asked, "Is not bacterial action essentially synthetic?" The limited knowledge at our command would, I believe, dictate the answer "Yes." If this question can be answered in the affirmative, our present system of media-making and culture study will need thorough revision; but before there can be much intelligent revision, further and more far-reaching investigations into the real requirements of a bacterial culture medium are indeed necessary.

We are told again and again that the so-called "parasitic" or "pathogenic" bacteria require a medium which simulates that of the natural animal host of these organisms. As a consequence of this prevailing idea fresh blood, blood agar, blood serum and ascitic fluid bouillon and agar, are regarded as indispensable in the isolation and cultivation of certain of the pathogens. In many instances sterilization by heat is avoided, in order that the natural nutrient properties may be preserved.

That these animal fluid media are valuable, and, in some instances, to all appearances necessary, cannot be denied. The common notion, however, that the peculiar nutrient properties are inseparably linked with the proteins as such, and that the value of the media depends upon these proteins, has little to support it. It is far more probable that the food-giving substances in the body fluids or tissues are nitrogenous substances of very simple constitution, perhaps of the nature of amines and amino acids. It must be assumed that in the profound chemical changes which take place in these fluids in the animal body, and in the process of autolysis after death or after their removal from the body, many and varied nitrogenous substances are formed which possess different degrees of stability. Some of the products are without doubt easily transformed into more stable substances, but, because of the extraordinary ease with which they are transformed, serve as excellent grist for bacteria, very much in the same way that a high grade of gasolene explodes more readily in the gasolene motor than one of much lower boiling point. Others, while less readily appropriated by the bacterial cell, are sufficiently unstable to be seized and utilized. The latter may in a large measure resist short periods of heat sterilization, while the more unstable intermediate products are destroyed in the process.

The extracts of animal organs, as well as those of some plant tissues, contain valuable nutrient material for bacteria which it is as yet impossible to supply in any medium of known chemical composition. Even commercial peptone possesses much value as an early stimulator of growth. This may be demonstrated readily on the diphtheria bacillus. The bacillus of Loeffler does not adapt itself to synthetic media. If but a very small amount of commercial peptone be added to the ordinary Uschinsky medium, however, growth soon takes place. Peptone is indeed a highly complex food, but it owes its value primarily to the substances of simple constitution which it contains. A peptone prepared without the application of heat in any stage of its preparation, if this were possible, would without doubt possess still greater value as a food for bacteria.

Fresh meat extract, also, is excellent nutriment for the bacterial cell. Commercial meat extract, while more uniform in composition than the other, loses much of its richness in the long process of preparation which involves prolonged application of heat and contact with filters.

The chemical composition of these more or less unstable but highly nutritive substances is a matter of purest speculation. For want of a better name they may well be termed "vitamins" or "accessory growth factors." While some are very unstable, others perhaps will withstand a certain amount of heat, and even rapid steam sterilization. The diphtheria bacillus seems to grow about as well on serum sterilized by the ordinary intermittent method as by the process originally advocated. It is a well known fact that *Treponema pallidum* will grow in ascitic fluid medium which has been sterilized by heat, and that, therefore, extreme precaution to use absolutely uncontaminated ascitic fluid is unnecessary. That there is danger of too strong application of heat is of course not to be denied.

The occurrence of vitamins in animal fluids, and probably in vegetable tissues, and their significance in the cultivation of the meningococcus, have received considerable attention by Dorothy Lloyd (1916) in her recent cultural studies of this organism. According to these investigations, primary cultivation of the meningococcus is possible only in the presence of certain accessory growth factors which are present in blood serum and other animal fluids, and probably in vegetable tissues. These accessory bodies are moderately heat stable, and are soluble in alcohol and in water. They are rapidly absorbed from solution by filter paper, but not by glass wool. The vitamins increase the reaction velocity of the proteolytic metabolism of the meningococcus. After the first or primary artificial cultivation the meningococcus gradually becomes independent of these substances. Old laboratory strains need no vitamins so long as amino acids are present. The main food requirements are the amino acids.

Cole and Lloyd (1917) have shown the following to be important factors in the cultivation of the gonococcus. Suitable H

ion concentration, preferably $P_H = 7.6$, high concentration of amino acids, and the presence of certain special "growth hormones." The importance of blood and other animal tissues is emphasized, but it is claimed that sterilization of the medium by heat does not destroy the hormones which favor the growth of the gonococcus.

Gordon, Hine and Flack (1916) call attention to the special growth-stimulating properties of pea flour and of wheat germ extract. The former favored early growth better than the germ extract, while the wheat germ extract had the more favorable influence on the longevity of the meningococcus. Agar containing pea flour extract deteriorated on autoclaving. The vitamins were not precipitated by alcohol.

Here is a field that is full of promise to the student of bacterial nutrition. That it is fraught with difficulties, some of them perhaps insurmountable, cannot be denied. Not only is there need of improved methods of cultivation in so far as the more difficultly grown bacteria are concerned, as for example the highly specialized parasitic or pathogenic organisms, but a thorough revision all along the line is of great importance.

The isolation and the enumeration of bacteria by the usual plate method have their serious defects, in spite of the marvelous progress in bacteriology which the method of isolation on solid media devised by Koch in 1880 stimulated. And in no field is the deficiency felt more keenly than in the isolation and study of the anaerobes. It may be said, in fact, that there is as yet no accepted method of enumerating anaerobic organisms, let alone the question of isolation. The difficulty encountered is not merely one of complete anaerobiosis, but fundamentally one of nutrition. The somewhat recent methods of cultivation advocated by Tarozzi, (1905) Calderini (1909) and others, strongly support this contention, though the interpretations or explanations of the influence of the pieces of fresh tissues in ordinary bouillon, by the open tube method, have never been clarifying. Is not the favorable influence on the anaerobes one of chemical or physiological stimulation by the peculiar growth-stimulating substances which certain animal tissues possess?

With optimum conditions as to nutriment, and absence of growth-inhibiting agents, the question of anaerobiosis is perhaps not as important as it was once thought to be.

So-called "bacterial lag" has its origin, in all probability, in a paucity or temporary absence of growth-stimulating substances in the new environment to which the organisms are transferred. Penfold has shown that the size of the inoculum is a factor. With larger amounts of material transferred corresponding amounts of the "intermediate bodies" are carried over. When the amounts of inoculum are very small the individual organisms are for a time at the mercy of the new medium, the customary intermediate bodies being absent, or present in such small quantities as to be of little or no assistance. The same reasoning should hold in so far as enzymes are concerned. We have on many occasions found that by very sparse seeding of a culture medium with a vigorous proteolytic enzyme-producing organism an indefinite or indeed permanent lag period was established, when the medium was one of definitely-known chemical composition to which purified protein had been added.

According to Penfold (1914) maximum growth presupposes optimum concentration of intermediate bodies attainable by the bacteria. When the bacteria cease developing, the intermediate bodies diffuse out or disappear. The presence of inhibitory products of metabolism of the organisms must always be taken into consideration, of course. A most satisfactory culture medium, or one that will of itself practically eliminate bacterial lag, will be a medium which furnishes satisfactory substitutes for the intermediate bodies, in the form of amino acids and perhaps amines of simple composition, and also certain growth accessory substances. When such an artificial medium is available our methods of enumerating and isolating bacteria by the plating process will be attended with much greater success than they are now. Too many individual bacteria fail to develop, when planted in high dilution, for want of proper environment and assimilable food, and die from inanition.

These are problems, you say, of the bio-chemist. Quite true; but the biochemist of today must be well grounded in general

chemistry, and particularly in organic chemistry. Unless the physiological chemist is also a bacteriologist, his interest will be in the field of animal or plant physiology. The chemist must have sufficient training in bacteriology to enable him to appreciate its many-sided problems and, unless he can rely on some one else, who is competent, he must be familiar with bacteriological technique. On the other hand, the bacteriologist is required to be well enough trained in the principles of chemistry, and especially organic and physiological chemistry, to master the situations that are sure to confront him. Thorough co-operation between the bacteriologist and well-trained chemist may at times be a possible solution, but the two will, as a rule, not have the same interests.

Bacteriology is still in its infancy. Numerous phases of applied bacteriology have been developed with amazing rapidity, and have led to many and important discoveries; but bacteriology as a science among other sciences is just beginning to emerge from its peculiarly chaotic state, and promises to offer him who approaches it with the right spirit and preparation a most fertile field for study and investigation.

REFERENCES

Armand-Delille et al. 1913. J. de Physiol. et de Path., **15**, 797–811.
Calderini, A. 1909. Centralbl. f. Bakteriol., **51**, Orig., 1 Abt., 681–5.
Chick, H., and Martin, C. J. 1908 J. Hyg., **8**, 654–97.
Cole, S. W. and Lloyd, D. 1917 J. Path. and Bacteriol., **21**, 267–86.
Dakin, H. D. et al. 1916 Proc. Roy. Soc. Lond., Series B, **89**, 232–51.
Gordon, Hine and Flack. 1916 Brit. Med. J., July–December, 678–82.
Lloyd, D. 1916 J. Path. & Bacteriol., **21**, 113–29.
Otsuka, I. 1916 Acta Scholae Med. Univ. Imp., Kioto, **1**,199–214. Physiol. Abstracts, London, **2**, 15.
Penfold, W. J. 1914 J. Hyg., **14**, 215–41.
Sasaki, T. 1912 Biochem. Ztschr., **47**, 463–71.
Tamura, S. 1913 Ztschr. f. physiol. Chem., **87**, 85–114; **88**, 190–8.
Tamura ,S. 1914 Ztschr. f. physiol. Chem., **89**, 289–311.
Tarozzi, G. 1905 Centralbl. f. Bakteriol., **33**, Orig., 1 Abt., 619–24.

METHODS OF PURE CULTURE STUDY

PRELIMINARY REPORT OF THE COMMITTEE ON THE CHART FOR IDENTIFICATION OF BACTERIAL SPECIES[1]

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The following methods have been prepared primarily to accompany the chart recommended at the 1917 meeting for use in instruction in bacteriology. So far as they apply, they should also be used for the older chart; but many of the determinations called for by the older chart have not yet been studied by the committee, and are not included in this report. It is the desire of the committee that these methods be put in provisional use during 1918, and that they be adopted as standard methods at the 1918 meeting, after such changes have been made in them as the year's use shows to be necessary. The chairman of the committee will be glad to receive suggestions in regard to such changes. Reprints of this report may be obtained from the secretary of the society at about cost price.

PREPARATION OF MEDIA

Beef-extract broth shall have the following composition:

Beef-extract	3 grams
Peptone	5 grams
Distilled water	1000 cc.

Beef-extract agar shall be of the same composition plus the addition of 12 grams of oven-dried agar or 15 grams of commercial agar. *Beef-extract gelatin* shall be of the same composition, with the addition of 120 grams of Gold Label gelatin, or 100 grams of some brand of gelatin (such as "Bacto-gelatin," or United States Glue Company gelatin) having greater jellying

[1] Presented at the Washington meeting of the Society of American Bacteriologists, December 28, 1917.

power. These media are to be made up according to the directions given by the committees of the American Public Health Association on water analysis and on milk analysis (1916, 1917), except that white of egg maybe used for clarification if desired. It is recommended, however, that instead of using phenolphthalein in adjusting the reaction of these media the simpler and more accurate procedure be adopted of adjusting to the neutral point of brom thymol blue[2]. Bring the media to such an acidity as to turn this indicator a distinct grass-green (neither yellow green nor blue green). This color indicates approximately "true neutrality," i.e., a hydrogen-ion concentration between $P_H = 6.6$ and $P_H = 7.4$, a variation which seems to have no appreciable effect on bacterial activities. Another equally satisfactory method of adjusting media to this hydrogen-ion concentration is to bring them to such an acidity as to cause the first faint trace of permanent pink to appear with phenol red.[3]

Sugar broths. Just before sterilization 1 per cent of the required carbohydrate is to be added to beef-extract broth. Otherwise proceed as for sugar-free broth. Adjust reaction with brom thymol blue or phenol red.

Plain gelatin. Proceed as for beef-extract gelatin, but omit beef-extract and peptone. Clarify with white of egg.

Nitrate broth. For routine work this medium should contain:

Peptone	1 gram
KNO_3	1 gram
Distilled water	1000 cc.

Filtering is unnecessary, and reaction requires no adjustment. Modification of this formula is necessary for some organisms (see p. 124).

Starch agar. Add 0.2 per cent of water-soluble starch to the ordinary beef-extract agar.

Indicator media. Saccharine media with some indicator to show acid production are frequently used. Litmus is the most

[2] 0.04 per cent di bromo thymol sulphonphthalein in 95 per cent alcohol.
[3] 0.02 per cent phenol sulphonphthalein in 95 per cent alcohol.

common indicator, enough of which should be added in saturated aqueous solution to give the medium a distinct blue color (taking care that the litmus solution used is not so alkaline as to alter, appreciably, the reaction of the medium). Litmus, however, does not give accurate results in terms of hydrogen ion concentration; so except for certain special purposes (see p. 124) it is recommended that brom cresol purple be used. Prepare this by dissolving 0.4 g. of di bromo ortho cresol sulphonphthalein in a minimum amount of alcohol, making up to 1 litre with water. Add 40 cc. of this solution to a litre of the medium. The blue or purple color given to media by this indicator begins to fade with the slightest production of acid in the medium and disappears completely at an acidity corresponding to $P_H = 5.0$, considerably below the curdling point of milk.

Variations of these media. For certain organisms the above formulae are not the best—many pathogenic bacteria, for instance, require more peptone than is provided in the above formula for broth, while some organisms are best studied in media of a hydrogen-ion concentration different from that recommended above. In such cases the individual investigator is free to modify them to suit his own purposes; but whenever other than these standard formulae are used, the fact should be stated on the chart. In employing a reaction other than that of neutrality it is recommended that instead of using the titrimetric method, the reaction be adjusted to some definite shade of brom cresol purple, if a more acid reaction is desired, or of phenol red if it is to be more alkaline.

Optional media. In many laboratories other media than those specifically mentioned on the chart are in general use, such as potato, blood serum, agar stabs, and so forth. Blank spaces are left on the chart for recording characteristics on any optional media.

INVIGORATION OF CULTURES

Provided a medium can be found upon which the organism to be studied grows vigorously, it should always be invigorated

before study, even though freshly isolated from its natural habitat. The procedure to employ is as follows:

Prepare duplicate sub-cultures in standard glucose broth, and on standard agar slopes, placing cultures of each at 37° and 25°C. On the basis of the resulting growth the organism falls into one of the following series:

Series I. Organisms which produce good growth (surface growth, distinct turbidity, or heavy precipitate) in twenty-four hours at 37° in glucose broth.

Series II. Organisms which do not produce good growth in twenty-four hours as above, but do in forty-eight hours at 25° in glucose broth.

Series III. Organisms which do not grow well in glucose broth but do produce good growth on the surface of agar in twenty-four hours at 37°.

Series IV. Organisms excluded from the above groups but which produce good growth on the surface of agar in forty-eight hours at 25°.

Record the series number on the chart at the proper place and proceed with the invigoration by inoculating into another tube of glucose broth for organisms of series I and II, or of standard agar for organisms of series III and IV. Incubate this tube at the temperature, and for the time, called for by the series in which it belongs; then transfer from this tube to a third tube and incubate as before. From this third culture make a gelatin or agar plate and incubate at the temperature previously used until colonies of sufficient size for isolation are obtained. Transfer from a typical colony to one or more agar slants and incubate one day at 37° or for two days at 25° according to the temperature relation of the organism studied.

In case the organism does not produce vigorous growth on either of these media at either temperature, it should be invigorated with any medium and at any temperature known to be adapted to its growth. Under such circumstances invigorate by the procedure just outlined but using the medium and temperature found most favorable for the organism in question, recording on the chart the method of invigoration adopted. If no

conditions are known under which the organism in question produces vigorous growth, it should be studied without preliminary cultivation as soon as possible after isolation from its natural habitat. Such an organism is not likely to give good growth on any ordinary media, and the results of the study called for by the chart will have little significance.

STUDY OF MORPHOLOGY

The routine study of morphology should be from dried preparations, stained with fuchsin, methylen blue, or gentian violet. Preparations to show the vegetative cells should be made, preferably, from agar slant cultures, from a few hours to two days old, according to the rapidity of growth. The medium and temperature used and the age of the culture should be recorded.

Motility. Hanging-drop preparations of young broth or agar cultures should be examined for motility. If motile, microscopic preparations should be made to show the arrangement of the flagella, using any of the ordinary methods of flagella staining with which the student can obtain good success. Even if motility is not observed in hanging-drop, it is wise to attempt a flagella stain, because motile organisms often lose their motility under the conditions of observation. Even negative results from both hanging-drop preparation and flagella stain do not absolutely prove that the organism is immotile.

Presence of spores. Routine examinations for spores should be made on stained, dried preparations from agar slant cultures a week old. Stain with methylen blue. Vegetative forms take the stain, but spores do not. In most cases there will be no trouble in finding spores if the organism produces them. All rather larger rods however, (0.8 micron or more in diameter) should be regarded as possible spore-producers, even though microscopic examination does not show spores. Such bacteria should be mixed with sterile water and heated to 85°C. for ten minutes; if still alive, spores may be regarded as unquestionably present. Also make repeated transfers of the culture onto agar

and examine at various ages. A culture of a large rod should not be recorded as a non-spore-former unless all these tests are negative.

Capsules. An organism should not be recorded as having capsules unless they have been actually stained by one of the methods of capsule-staining described in bacteriological text books.

Irregular forms. Forms that differ from the typical shape for the organism (i.e., "involution forms," etc.) such as branching forms, clubs, spindles or filaments should be noted and sketched.

Special stains. In making the Gram or Neisser stain and in testing the acid-fast properties of the organism, directions given in any reliable laboratory manual may be followed.

Sketches. Drawings of all the morphological characteristics should be made on the blank spaces on the chart to the right of the descriptions. Both typical and atypical forms should be sketched, using care to designate which are typical.

CULTURAL CHARACTERISTICS

Cultures for the study of cultural characteristics should be incubated at 37°C. in case of organisms of series I and III, and at 25° in case of organisms of series II and IV, except that gelatin cultures should be incubated at 20°. Room temperature may be used in place of 25° at certain seasons of the year; but if a minimum thermometer shows that the temperature falls below 22° during the course of the work, note should be made of the fact. On the day when good growth first appears the proper descriptive terms on the card should be underlined; after subsequent study, the changes should be noted in the space provided, and sketches of the different stages should be made.

PHYSIOLOGY

Liquefaction of gelatin. Old method. The method in most common use is to hold gelatin stab cultures six weeks at 20°C. Plain gelatin should be used.

Provisional method. It is recommended that the following method proposed by Rothberg (1917) be put in provisional use until experience shows its value. It is designed to distinguish "true liquefiers" (organisms producing ecto-enzymes) from the organisms that produce endo-enzymes of proteolytic action that are released from the cell after death and cause liquefaction of the gelatin if incubated for the long period mentioned above. The method is to give the organism a preliminary cultivation for eighteen to forty-eight hours (according to its rapidity of growth) in a 1 per cent solution of gelatin at 25° or 37° according to its temperature relations; then inoculate on surface of gelatin in test tube and incubate 15 days at 20°.

Relation to free oxygen. Provisional method. Determine by noting the presence or absence of growth in open and closed arm, respectively, of fermentation tubes containing glucose broth. Care must be taken to use fermentation tubes from which the dissolved oxygen has been recently driven off by heating. In case of gas production, this test is of comparatively little value, because bubbles of gas may carry the sediment up with them; hence if an organism produces gas from glucose, the test should, if possible, be made in the presence of some other sugar which it attacks (acidifies) without gas-formation. It must be remembered, however, that even anaerobes do not grow in the absence of free oxygen except in the presence of a chemical substance (such as carbohydrate) which they are able to reduce and use as a source of oxygen.

Fermentation of sugars and glycerin. This is normally to be studied in fermentation tubes. Ordinarily use beef-extract broth containing 1 per cent of the substance investigated; but if the organism does not grow well in such broth and some medium is known in which it does grow well, the latter may be used. Generally speaking, organisms of series I and II should be studied in broth, organisms of series III and IV in some other medium. Incubate organisms of series I and III at 37°, organisms of series II and IV at 25°. Test ordinarily on 1st, 3rd, and 7th days, although the best days for testing will depend upon the rapidity of growth of the culture. Inoculations should always be made at least in triplicate.

To test for acid, it is recommended that in place of the illogical titrimetric method, determinations of hydrogen-ion concentration be made by the colorimetric method described by Clark and Lubs (1917a). In accurate research work the exact shade of the indicator should be compared with that obtained in standard "buffer" solutions, and results recorded in terms of P_H. In laboratories where these standard solutions cannot be obtained, it is better to record results simply as + or −, according to the reaction of the culture to litmus, than to use the titration method. Under such conditions it is possible, however, to obtain a rough idea of the hydrogen-ion concentration by the use of Clark and Lubs' series of indicators without making accurate determinations of P_H. Four different degrees of acidity can be easily distinguished by this simple method in sugar broth with initial reaction of neutrality. The indicator reactions for these different degrees of acidity are listed in table 1, together with the approximate range of P_H to which each corresponds. In the absence of accurate determinations, these degrees of acidity may be recorded by the indefinite terms, "weak," "moderate," "strong" and "very strong," or by the symbols +, ++, +++, and ++++.

Gas production is ordinarily measured in percentage of gas in the closed arm, and the ratio of $H:CO_2$ by means of absorption with NaOH, using the methods described in laboratory manuals (filling open arm with 4 per cent NaOH, allowing gas to enter open arm, shaking and returning gas to closed arm). As this method is far from accurate, it is recommended for provisional use only.

The fermentation test is ordinarily of no significance for organisms of series III and IV because of their poor growth in broth. Sometimes these organisms can be studied in some other liquid medium in which they do give good growth; but often it is necessary to use agar slants. In such a case, use a sugar agar containing brom cresol purple (see p. 117). Litmus can be used, but is unsatisfactory. Often gas-production can be detected in agar cultures by the presence of cracks and air bubbles but, as a test for gas, agar slants are not as reliable as fermentation tubes.

Milk. Acid production in milk can be detected by adding brom cresol purple to the culture and comparing with the color obtained by adding the same proportionate quantity of indica-

TABLE 1

Degrees of acidity easily recognized in clear media

ACIDITY	INDICATOR REACTIONS	APPROXIMATE PH-VALUE
"Neutral"........	Blue or green to brom thymol blue*	Over 6.2
"Weak"........	Yellow to brom thymol blue Purple to brom cresol purple*	5.2–6.0
"Moderate"....	Yellow to brom cresol purple Orange to methyl red†	4.6–5.0
"Strong".......	Maximum red to methyl red Blue or green to brom phenol blue*	3.2–4.4
"Very strong"....	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.
† Use a 0.02 per cent alcoholic solution.

TABLE 2

Degrees of acidity easily recognized in milk

ACIDITY	INDICATOR REACTION, ETC.	APPROXIMATE PH-VALUE
"Neutral"........	Same color with brom cresol purple* as sterile milk; i.e., blue to gray-green	6.2–6.8
"Weak"..........	Color with brom cresol purple lighter than in sterile milk; i.e., gray-green to greenish yellow	5.2–6.0
"Moderate"......	Yellow with brom cresol purple. Not curdled	4.7–5.0
"Strong".........	Curdled. Blue or green to brom phenol blue*	3.2–4.6
"Very strong"....	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.

tor to sterile milk. (Brom thymol blue does not give satisfactory results in milk.) Four degrees of acidity that can be simply recognized in milk are listed in table 2. They correspond closely to those listed in table 1, differing only in that brom

cresol purple is used instead of brom thymol blue to show "neutrality" and that the curdling point ($P_H = 4.7$) is used to separate between "moderate" and "strong" acidity instead of the less definite point of maximum red to methyl red. The same methods of expression used in recording acidity in clear media should be used in recording that of milk.

Litmus milk often gives valuable information, showing not only the production of acid, but also decolorization of the litmus by organisms that are able to reduce it. More accurate results as to acidity can be obtained by using brom cresol purple, as shown by Clark and Lubs (1917b). This indicator, however, does not show the reduction phenomena which are sometimes of diagnostic value in litmus milk cultures; so its substitution for litmus is not always to be recommended.

Reduction of nitrates. For routine work, nitrate broth should have the composition given on p. 116. Always use this broth first unless the organism is known to grow poorly in it. If an organism does not give good growth in this medium (as indicated by distinct cloudiness and precipitate), a negative nitrite test in this medium is meaningless. In such a case record the results of the test as doubtful, putting a question mark in the group-number at the point which indicates action on nitrates; or else make the test in some nitrate-containing, nitrite-free medium in which the organism does produce good growth. For instance, if (like *B. communis* and most pathogens) it requires considerable organic matter, use 2 or even 5 grams of peptone per litre; or if it grows poorly in the presence of organic matter, use some nitrite-free synthetic medium in which it is known to grow. The tubes should be inoculated in triplicate and incubated at 37° for organisms of series I and III, at 25° for organisms of series II and IV.

To test for nitrite the following reagents are necessary: (1) dissolve 8 grams sulphanilic acid in 1 litre of 5N acetic acid (1 part glacial acetic acid to 2.5 parts of water) or in 1 litre of dilute sulphuric acid (1 part concentrated acid to 20 parts water); (2) dissolve 5 grams α-naphthylamine in 1 litre of 5N acetic acid or of very dilute sulphuric acid (1 part concentrated acid

to 125 parts water). Add a few drops of each of these reagents to 3 to 5 cc. of the culture to be tested. A distinct pink or red indicates the presence of nitrite. Always test a sterile check that has been kept under the same conditions, to guard against errors due to the absorption of nitrite from the air.

Gas is rarely produced from nitrates, and when produced is generally to be observed as bubbles in an open test tube. If its presence is suspected, however, the culture should be tested in a fermentation tube.

In interpreting results, it must be remembered that the absence of nitrite in a culture showing poor growth does not indicate that the organism cannot reduce nitrates. If nitrate reduction is observed in any medium whatever, the organism is to be recorded as a nitrate-reducer; but unless the routine formula is used, the exact composition of the medium must always be given.

Chromogenesis. Color production should be recorded if observed in broth, on beef-extract agar, gelatin or potato, or if noticed to a striking extent on any other medium. In the group number, the point devoted to chromogenesis refers to the color produced on beef-extract agar.

Diastatic action on starch. Provisional method. Use beef-extract agar containing 0.2 per cent of soluble starch. Pour into a petri dish, and after hardening make a streak inoculation on its surface. Incubate at 37° for organisms of series I and III, at 25° for organisms of series II and IV. Determinations for the group number shall be based upon results obtained on the seventh day. To make the test, flood the surface of the petri dishes with a saturated solution of iodine in 50 per cent alcohol. The breadth of the clear zone outside of the area of growth indicates the extent of diastatic action. If over 2 mm. in width on the seventh day it shall be recorded as "strong;" if under 2 mm. as "feeble;" if no clear zone is present, as "absent."

This method is advised by P. W. Allen (1918) and, although it is the best method yet called to the attention of the committee, it is recommended only for provisional use. It requires some

modification e.g., reducing the amount of peptone in the medium, for organisms that grow so rapidly as to cover the entire surface of the plate in seven days, thus leaving no room for a clear zone outside.

The group-number is a brief means of recording the salient features of the organism. It is primarily a summary of the physiological characteristics just discussed. As each of the determinations is made, the proper figure for that place in the group number is to be checked or underscored. After completing the determinations, the entire group number is to be written in at the place left for it on the chart. The genus symbol should precede the group number. The present group number, adopted by the Society in 1907, is intended for use with the generic names of Migula. As Migula's genera are not in such general use today as they were ten years ago, a revision of the group number on some other basis will be necessary in the near future.

Brief characterization. On the right hand margin of page one of the chart is a place for recording by a + or − sign other important characteristics of the organism (primarily cultural) not included in the group number. This margin together with the group number constitute a brief characterization of the organism,—a summary of the tests outlined above.

REFERENCES

Allen, P. W. 1918 A simple method for the classification of bacteria as to diastase production. J. Bact. **3**, 15–18.

American Public Health Association. 1916 Standard methods for the examination of water and sewage.

American Public Health Association 1917 Standard methods of bacteriological analysis of milk. Am. J. Pub. Health, **6**, 1315–1325.

Clark, W. M. and Lubs, H. A. 1917 a The colorimetric determination of hydrogen-ion concentration. J. Bact. **2**, 1–34, 109–136, 191–236.

Clark, W. M. and Lubs, H. A. 1917 b A substitute for litmus for use in milk cultures. J. Agric. Research, **10**, 105–111.

Rothberg, W. 1917 Observations on some methods for the study of gelatine liquefaction. Read before Soc. Amer. Bacteriologists, December, 1917.

Glossary of Terms used on the Chart

Adherent, Applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen; **strictly aerobic**, growing *only* in the presence of free oxygen.

Amorphous, without visible differentiation in structure.

Anaerobic, growing in the absence of free oxygen; **strictly anaerobic**, growing *only* in the presence of free oxygen; **facultative anaerobic**, growing both in presence and in absence of free oxygen.

Arborescent, branched, tree-like growth.

Beaded, in stab or stroke culture, disjointed or semi-confluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Brittle, growth dry, friable under the platinum needle.

Butyrous, growth of butter-like consistency.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Ciliate, having fine, hair-like extensions, resembling cilia, sometimes not visible to the naked eye.

Clavate, club-shaped.

Coagulation, the separation of casein from whey in milk.

Contoured, an irregular, smoothly undulating surface, like that of a relief map.

Convex, surface the segment of a sphere.

Crateriform, a saucer-shaped liquefaction of the medium.

Cuneate, wedge-shaped.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.

Diastatic action, conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.

Echinulate, a growth along line of inoculation with toothed or pointed margins.

Effuse, growth thin, veily, unusually spreading.

Endospores, thick-walled spores formed within the bacterial cell; i.e., typical bacterial spores like those of *B. anthracis* or *B. subtilis*.

Entire, with an even margin.

Erose, border irregularly toothed.

Filaments, applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented, to be distinguished from chains (q. v.) by the absence of constrictions between the segments.

Filamentous, growth composed of long, irregularly placed or interwoven threads.

Filiform, in stroke or stab cultures, a uniform growth along line of inoculation.

Flocculent, containing small adherent masses of bacteria of various shapes floating in the culture fluid.

Fluorescent, having one color by transmitted light and another by reflected light.

Granular, composed of small granules.

Infundibuliform, form of a funnel or inverted cone.

Iridescent, exhibiting changing rainbow colors in reflected light.

Lobate, having the margin deeply undulate, producing lobes (see *undulate*).

Luminous, glowing in the dark, phosphorescent.

Maximum temperature, temperature above which growth does not take place.
Membranous, growth thin, coherent, like a membrane.
Minimum temperature, temperature below which growth does not take place.
Mycelioid, colonies having the radiately filamentous appearance of mold colonies.
Napiform, liquefaction in form of a turnip.
Opalescent, resembling the color of an opal.
Optimum temperature, temperature at which growth is most rapid.
Papillate, growth beset with small nipple-like processes.
Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.
Peptonization, rendering curdled milk soluble by the action of trypsin.
Peritrichiate, covered with flagella over the entire surface.
Persistent, lasting many weeks or months.
Plumose, a fleecy or feathery growth.
Polar, at the end or pole of the bacterial cell.
Pulvinate, decidedly convex, in the form of a cushion.
Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.
Radiate, showing ray-structure.
Raised, growth thick, with abrupt or terraced edges.
Reduction, removing oxygen from a chemical compound. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen, and to the decolorization of litmus.
Rhizoid, growth of an irregular branched or root-like character, as in *B. mycoides*.
Ring, growth at the upper margin of a liquid culture, adhering to the glass.
Rapid, developing in 24 to 48 hours.
Rugose, wrinkled.
Saccate, liquefaction in form of an elongated sac, tubular, cylindrical.
Slow, requiring 5 or 6 days for development.
Spindled, larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called *clostridia*.
Sporangia, cells containing endospores.
Spreading, growth extending much beyond the line of inoculation, i.e., several millimeters or more.
Stratiform, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.
Transient, lasting a few days.
Truncate, ends abrupt, square.
Turbid, cloudy with flocculent particles; i.e., cloudy plus flocculence.
Umbonate, having a button-like, raised center.
Undulate, border wavy, with shallow sinuses.
Viscid, growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.

THE ENZYMES OF THE TUBERCLE BACILLUS

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The tubercle bacillus and the related acid fast bacilli (lepra, smegma, etc.,) have for a great many years been looked upon as distinct and different organisms, biologically and chemically, from the ordinary rapidly growing bacteria, and it is rather singular that until recently the tubercle bacillus should have been considered not to possess the ordinary well-known enzymes. This lack of knowledge as to the presence of enzymes in the tubercle bacillus is, of course, explicable on the basis that not until within recent years have sufficiently delicate quantitative methods been available for the demonstration of these interesting catalytic substances in such a sluggish organism as the tubercle bacillus and in amounts so small or of so slight an activity as those characteristic of this organism. It is worthy of note also that these problems could not have been solved before the days of the use of antiseptics in studying enzymatic action. During the course of investigations carried out to find a suitable antigen for use in complement fixation tests for tuberculosis, it was deemed advisable to understand more fully the part played by the enzymes in the tubercle bacillus and for this purpose the experiments to be reported upon were carried out.

LITERATURE

In order to study the action of the enzymes of various bacteria upon different substances and thus gain a better insight into the nature of these enzymes, Eijkman (1901) used a simple plate method called the "auxanographic" method of Beijerinck or the diffusion method of Wijsman. This method, which consists in mixing various substrates (casein, blood, starch, tallow,

cellulose, etc.,) with nutrient agar and observing the effect of the growths of various organisms upon the substrates, was of necessity crude and gave only gross results. The criterion of enzymatic action was merely the formation of a zone of clearing or change in the media surrounding the colonies. Thus Eijkman found that the following gelatin liquefying or digesting organisms also cause the solution of casein: *B. anthracis*, *B. pyocyaneus*, *Staphylococcus pyogenes aureus*, *B. Metschnikowi*, *B. cholerae*, *B. fluorescens*, *B. prodigiosus*, *B. indicus*, *B. ruber*, *B. subtilis*, *B. megatherium*, and *B. mesentericus*; while the following organisms, not peptonizing gelatin, also failed to dissolve casein: *B. typhosus*, *B. coli-communis*, *B. mallei*, *B. pestis*, *B. diphtheriae* and *B. lactis cyanogenes*. He concludes, therefore, that the same enzyme probably digests both substances.

As the hemolytic action of a number of bacteria (*B. diphtheriae B. anthracis*, *B. fluorescens*, *B. mesentericus*, *B. prodigiosus* and *B. indicus*) did not vary hand in hand with the gelatin liquefying property, he concludes that the hemolytic and tryptic enzymes are not identical, but suggests that different tryptic enzymes may be present in different bacteria and these might in some cases attack blood or gelatin more vigorously. Varying grades of diastatic action by pathogenic organisms were observed with starch. *B. anthracis* and *B. cholerae* intensely digested it, while *B. diphtheriae* and *B. dysenteriae* (Kruse) had only a faint action and *B. mallei*, *B. pyocyaneus* and *Staphylococcus pyogenes aureus* were negative in this respect. Fat splitting power was also found to be present in some organisms and absent in others. None of those forms tested were able to split beeswax. In all tests with inulin, (Eijkman, 1913) keratin and cellulose negative results were obtained. Elastic tissue prepared from calf lung was digested by *B. pyocyaneus* (culture and bouillon filtrate),—which action was destroyed at 80°C.,—*B. anthracoides* and a bacillus isolated from a case of lung gangrene. It was noteworthy that all the elastic tissue dissolving organisms liquefied gelatin, but the reverse did not hold true.

Fontes (1911) was unable to demonstrate enzymes, zymases or oxidases in cultures or tuberculins of bovine and human tubercle

bacilli. Proteolytic enzymes were also not found in tuberculous pus free from other organisms. Opie and Barker (1908) had earlier observed that tuberculous tissues in the early stages contained a proteolytic enzyme acting in neutral and weakly acid solution. With the advance of caseation the one acting in alkaline solution disappears while the other (lympho-protease) is retained. After complete caseation the latter also disappears. No enzymes were demonstrable in tuberculous exudates in human beings (Opie and Barker, 1909).

Gosio (1905) using the tellurite and selenite reduction test devised by him was able to demonstrate reductases in all the bacteria tested including the various tubercle bacilli: avian, bovine and human. He examined 173 microorganisms and divided them into three classes, dependent upon the reaction obtained: (1) a decided reaction, (2) a less intense, but fully evident reaction (in this class he included a bovine, a human and an avian tubercle bacillus and a so-called pseudo-tubercle-bacillus (Rabinowitsch)), (3) a very slight reaction.

Wells and Corper (1912) studying the lipase of *B. tuberculosis* and other bacteria noted that toluene would kill the tubercle bacillus but did not destroy the lipases, and that lipolytic enzymes are present with different activites in the organisms tested (*B. dysenteriae, Staphylococcus pyogenes aureus, B. pyocyaneus, B. coli* and *B. tuberculosis*). Of these the tubercle bacillus was least actively lipolytic. They also noted that the "auxanographic" method was not suitable for use in studying the enzymes of the tubercle bacillus.

Kendall, Day and Walker (1914c) verified and elaborated these findings noting that various strains of the human tubercle bacillus, the bovine and avian bacilli as well as the leprosy, smegma and grass bacilli, form lipase during their growth on glycerin broth. This lipase is present in the medium free from bacteria. The bodies (Kendall, Day and Walker, 1914d) of acid fast bacteria grown in nutrient broth, with glucose, mannite and glycerin as additional sources of carbon, freed from adherent media, also contained a lipase not as active or as great in amount as that in the culture media. The authors were

unable to determine whether the lipase was freed as the result of autolysis or whether it was excreted as an exo-lipase.

The above authors (Kendall, Day and Walker, 1914d) also observed that the metabolism of the smegma and grass bacillus resembles that of the rapidly growing human tubercle bacillus in two important particulars; neither glucose, mannite nor glycerin exhibits any sparing action for the protein constituents as measured by the ammonia content of the broth (the "lepra bacillus" does not present this metabolic phenomenon); and a rapidly growing strain of human tubercle bacilli grown in a medium of very simple composition consisting essentially of diammonium hydrogen-phosphat, as a combined source of nitrogen and phosphorus, and glucose, mannite and glycerin respectively, as a source of carbon, so changed this medium that 10 per cent of the ammonium nitrogen had disappeared at the end of two weeks apparently being built up into bacillary bodies. At the end of four weeks between 40 and 50 per cent of the lost ammonia nitrogen reappeared in the media. This occurred coincidently with the cessation of vegetative activity, strongly suggesting, as stated by the authors, that it is associated with a certain amount of autolysis of the bacilli, (Kendall, Day and Walker, 1914a).

That the tubercle bacilli themselves contain enzyme inhibiting substances was shown by Jobling and Peterson (1914) who prepared unsaturated fatty acid soaps from tubercle bacilli which were more actively inhibitory than soaps prepared from linseed, olive and cod liver oils.

In the following investigations it will be observed that an attempt was made to check the findings obtained in every case by as many methods as possible. Frequent failures marked the progress of the work however, because certain of the methods used were not delicate enough for the purpose and others could not be used on account of interfering (absorption and turbidity) phenomenon. This was especially true of the nephelometric methods tried.

AUTOLYTIC ENZYMES

Method

The amount of non-coagulable nitrogen was determined in all the following experiments, except where otherwise indicated, by the colorimetric micro-method as devised by Folin and Farmer (1912) using the distillation modification of Bock and Benedict, (1915). The coagulable nitrogen was removed by the addition of ten volumes of 2.5 per cent trichloracetic acid according to Greenwald (1915). The results obtained were plotted in curves, using the initial nitrogen found as the zero point, that is the amount of non-coagulable nitrogen obtained in a definite volume (1 cc. of solution) at the beginning of the experiment was subtracted from the amount of nitrogen in an equivalent amount (1 cc.) of solution subsequently.

The method used for determining quantitatively the amount of amino acid α nitrogen was that devised by Harding and MacLean (1915; 1916). Asparagin was used as a standard and since the results are merely comparative the objections to the use of asparagin raised by the above authors did not apply to the procedure.

EXPERIMENTAL PART

Series I. Liberation of nitrogenous substances by tubercle bacilli suspended in salt solution

In order to determine whether there was a liberation of non-coagulable nitrogenous substances from tubercle bacilli under certain conditions, heavy suspensions were made of virulent human tubercle bacilli in sterile physiological salt solution and divided among three graduated centrifuge tubes in as nearly equal amounts as possible (each tube containing 10 cc. of heavy suspension). At the conclusion of the experiment the amount of residual nitrogen remaining in the bacilli was always determined and found to agree fairly well within the limits of error of the method, thus serving as a check upon the use of approxi-

mately equivalent amounts of bacilli in the three tubes. One of these tubes was heated as a control and kept in the incubator, one was placed in the incubator at 37°C., without previous heating and the third was kept at room temperature during the course of the experiment in order to determine the most favorable condition suitable for the liberation of the nitrogenous substances from the bacilli.[1]

It was found that tubercle bacilli suspended in physiological salt solution in this way liberate non-coagulable nitrogenous substances at incubator temperature (37°C.), but that this liberation requires a number of days. At room temperature (i.e., about 15° to 20°C.) this does not occur to any appreciable extent within the same time.

Series II. Determination of an autolysis of human tubercle bacilli

In order to determine whether the non-coagulable nitrogenous substances liberated at incubator temperature are merely dissolved out from the bacillary bodies of these substances or whether they are formed as a result of enzymatic action as suggested by Kendall, Day and Walker, tubercle bacilli in equal amounts in two tubes were suspended in sterile physiological salt solution and one was incubated at 37°C., as a control, while to the other was added 3 cc. toluene before incubation. The toluene, as has been shown by Wells and Corper (1912), kills the bacilli but leaves the enzymes intact.[2]

It appeared that there was a definite liberation of non-coagulable nitrogen in both tubes, rather more rapid and reaching a maximum earlier under toluene than in sterile physiological salt solution. A suggested explanation of the difference may be found in the fact that the bacilli are killed rapidly by the toluene and therefore more rapidly disintegrate as a result of enzymatic action, an autolysis.

[1] Charts depicting these results and those of Series II have been published in the Jour. of Infectious Diseases, Vol. 19, 1916, p. 315–21.

[2] See note under series I.

Series III. Determination of an autolysis of bovine tubercle bacilli

The experiments of series I and II were performed with human tubercle bacilli; this series is in a certain sense a repetition and check on the above except for the fact that bovine tubercle bacilli were used. These results are plotted in chart I.

They bear out the findings with the human tubercle bacilli in that the autolysis occurs more rapidly under toluene (antiseptic) than in merely aseptic conditions.

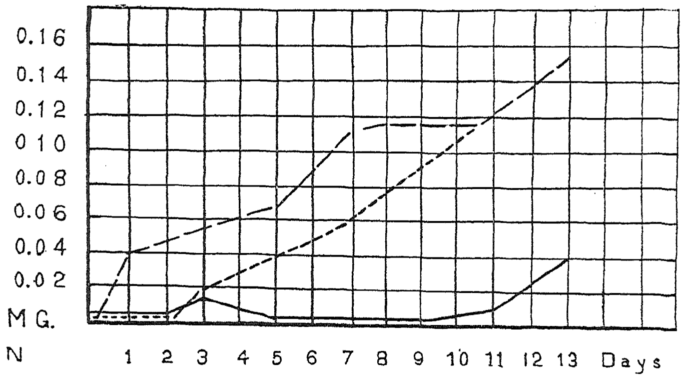

CHART I. AUTOLYSIS OF TUBERCLE BACILLI NON-COAGULABLE NITROGEN CURVES

Continuous line is heated control at incubator temperature. Dotted line is aseptic autolysis at incubator temperature. Dash line is antiseptic (toluene) autolysis at incubator temperature.

Series IV. Liberation of amino acids during autolysis of tubercle bacilli

As a check upon the above findings and in order to see whether the increase in non-coagulable nitrogen runs parallel with an increase in liberated amino acid α nitrogen, the above experiments were repeated and the autolysate was tested, at various intervals during incubation, for α amino acid content by the

Harding and MacLean colorimetric method. The results are plotted in chart II.

It is to be noted again that even though equivalent amounts of human tubercle bacilli were used in each test the curve for the antiseptic (toluene) autolysis is higher than the aseptic curve and that autolysis as indicated by the liberation of amino acids is more rapid at incubator (37°C.) than at room tempera-

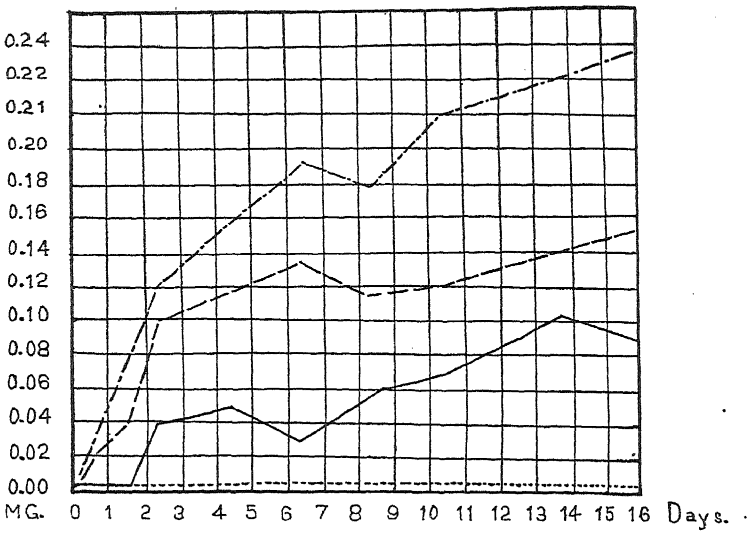

CHART II. AUTOLYSIS OF TUBERCLE BACILLI. AMINO ACID CURVES

Solid line is aseptic autolysis at room temperature. Dotted line is heated control. Long dash line is aseptic autolysis at incubator temperature. Dash dot line is antiseptic (toluene) autolysis at incubator temperature.

ture (these experiments were carried out in a warmer room than those reported in series I which probably accounts for the greater height attained by the room temperature curve).

Series V. Liberation of antigenic substances during autolysis

In order to determine whether, coincident with the autolysis of tubercle bacilli occurring at incubator temperature, there was

also an increase in soluble tuberculosis antigen in the autolysate available for complement fixation tests, that is capable of uniting with tuberculosis antibody, human and bovine tubercle bacilli were suspended in sterile physiological salt solution and kept in the incubator at 37°C. At definite intervals the clear autolysate was tested for non-coagulable nitrogen content and titrated for its antigenic strength. The antigenic titre was obtained by determining the minimum amount necessary to give a complete binding of all the complement when using a definite amount of + + + + antituberculosis (human) serum. The Noguchi antihuman hemolytic system was used in these tests. The results are tabulated in Chart III.

	Nitrogen Curves	Antigen Curves							
Days	M G.N. PER.C C	0.2 C C	0.1 C C	0.5 CC	0.01CC	0.005CC	0.001CC	0.0005CC	0.0001CC
0	0.00	++++	++++	+++	—	—	—	—	—
1	0.00	++++	++++	+++	±	—	—	—	—
2	0.01	++++	++++	++++	+++	±	—	—	—
3	0.02	++++	++++	++++	++++	+++	+ +	+	—
4	0.03	++++	++++	++++	++++	++++	+++	+	±
6	0.05	++++	++++	++++	++++	++++	++++	+ +	+ +
8	0.06	++++	++++	++++	++++	++++	++++	+ + +	+ +
10	0.08	++++	++++	++++	++++	++++	++++	+++	+
13	0.15	++++	++++	++++	++++	++++	++++	+ + +	+ +

CHART III. CORRELATION OF AUTOLYSIS AND ANTIGEN FORMATION

It is to be noted that coincident, although not absolutely parallel, with the increase in non-coagulable nitrogen liberated there also occurs an increase in the antigenic titre of the autolysate, so that an autolysate which only gave a titre of 0.1 cc. on the first day gradually and consistently increases to a titre of 0.001 cc. on the sixth day.

DETERMINATION OF INDIVIDUAL ENZYMES OF THE TUBERCLE BACILLUS

In the hope of being able to determine the presence of the various individual enzymes in the tubercle bacillus by the nephelometric method for determining enzymatic action recently

described by Kober and his colleagues (Graves and Kober, 1914; Kober, 1913; Kober and Graves, 1914), a series of experiments were carried out in which heavy emulsions of tubercle bacilli were mixed with definite amounts of various substrates (sodium caseinate for trypsin, acid casein for erepsin, acid edestin for pepsin, nucleic acid for nucleases, starch for diastase, sucrose for invertase, and urea for urease). In the majority of cases this method was found impractical, however, on account of the adsorption of the protein substrates by the tubercle bacilli, which made quantitative determinations impossible. In a number of instances this difficulty was, however, overcome by determining the presence of the enzymes in the autolysate where no adsorption occurs and checking their presence by other methods.

Methods

The methods used for quantitative nephelometric determination of the various substrates (casein, edestin and nucleic acid) were those described by Kober and his colleagues, the previously cited micro-method of Folin, modified by Bock and Benedict, for determining non-coagulable nitrogen, and the amino acid α nitrogen method of Harding and MacLean. The presence of urease was determined by the aeration method of Folin (Folin and MacCollum, 1912) and the presence of diastase and invertase by the amount of glucose liberated from starch and sucrose by the recent picramic acid method of Lewis and Benedict (1915) as used by Myers (1916) and Myers and Rose, (1916), for determining the presence of these enzymes in ptyalin and the presence of glucose, sucrose, dextrin and starch in foodstuffs. The quantitative Fehling method proved far too inaccurate and not delicate enough for this purpose.

Series I. Proteolytic enzyme acting in alkaline solution (trypsin-like enzyme)

Experiment 1. By its presence in the autolysate. (a) In a graduated 15 cc. centrifuge tube was emulsified 2 cc. of human tubercle bacilli and sufficient sterile physiological salt solution

was added to make a volume of 12 cc., and, as antiseptic, 2 cc. toluene and 1 cc. chloroform. After thoroughly mixing, this was placed in the incubator at 37°C. for twenty-four hours, centrifugated at the end of that time and the supernatant autolysate withdrawn, filtered through a hard filter paper and divided into two equal parts; one part heated for thirty minutes at 100°C., as control to destroy the enzymes, and the other half kept intact. To each was added 1 cc. 0.1 per cent sodium caseinate, sodium carbonate to make 0.3 per cent, 1 cc. toluene and 1 cc. chloroform. The tubes containing these mixtures were then incubated at 37°C., and a definite amount of the clear watery solution withdrawn at various intervals and compared, control and test, nephelometrically after precipitation with sulphosalicylic acid. The results obtained, figured in percentage of the heated control, were as follows:

	0	2 DAYS	4 DAYS	6 DAYS	12 DAYS
Percentage of original casein solution added...	100	82.2	79.6	78	63

The above figures indicate a definite splitting of the casein by the autolysate withdrawn at one day.

(b) The above experiment 1a was repeated but the autolysate was withdrawn after twelve days in the incubator at 37°C. The results obtained with the autolysate as compared to the heated control were:

	0	3 DAYS	6 DAYS	9 DAYS	12 DAYS
Percentage of original casein solution added...	100	80	74.4	73	71.4

These figures indicate a definite hydrolysis of the casein by the autolysate withdrawn at twelve days.

(c) In order to check this point more fully a more complete experiment was performed. Human tubercle bacilli, 2 cc. by volume, were suspended in physiological salt solution to make a total volume of 12 cc., and 2 cc. toluene and 1 cc. chloroform added. The mixture was incubated for three days, centrifu-

gated and about 10 cc. of autolysate withdrawn. This was then replaced by physiological salt solution and the entire tube incubated another three days. The same process was again repeated at nine and twelve days. The autolysate after the various intervals of three, six, nine and twelve days, was divided into two equal portions (1 portion to be heated for control and the other to be retained intact). To each of these was added

TABLE 1

Period of determination after adding substrate

AGE OF AUTOLYSATE		THREE DAYS	SIX DAYS	NINE DAYS
		per cent	*per cent*	*per cent*
Three days	T*	109	94	68
	H	125	125	121
Six days	T	83	87	84
	H	97	106	107
Nine days	T	89	83	87
	H	97	100	103
Twelve days	T	91	86	87
	H	98	97	98

* T designating the test for enzyme and being the percentage of casein recovered as compared with a standard containing the amount of casein originally added; H, designating the percentage of casein in the heated control as compared with the same standard containing the amount of casein originally added. The data are given in this form to rule out any conflicting turbidity or sources of error which may have been occasioned by the autolysate itself.

When these experiments were performed it was not realized that there were any conflicting turbidities formed or other sources of error and so a test sample was not withdrawn immediately, it being taken for granted that the heated control would suffice for this purpose.

1 cc. 0.1 per cent sodium caseinate, enough sodium carbonate to make 0.3 per cent, 1 cc. toluene and 1 cc. chloroform. This mixture was incubated at 37°C., and a definite amount withdrawn after definite intervals of incubation (three, six and nine days) and after precipitation with sulphosalicylic acid tested nephelometrically for casein content. The results are given in table 1.

These results indicate a definite splitting of the casein by the autolysate withdrawn at the third day of autolysis, but subsequently the enzymes have either been entirely washed away from the bacilli or have been so diluted by the additional salt solution added that they are incapable of exerting any action upon the casein.

Experiment II. By the non-coagulable nitrogen liberated in alkaline solution. Tubercle bacilli, 4 cc. of a heavy emulsion, were placed in 15 cc. graduated centrifuge tubes (2 cc. in each). One as control was heated to kill the bacilli, and to both was added 10 cc. physiological salt solution, sufficient sodium carbonate to make 0.3 per cent, 2 cc. toluene and 1 cc. chloroform. The tubes were then placed in the incubator at 37°C., after withdrawing 1 cc. as a control test, and 1 cc. of the clear supernatant solution was withdrawn after centrifugation daily, for eight days. The non-coagulable nitrogen was determined as before by the Folin micro-method.

	DAYS									RESIDUAL NITROGEN
	0	1	2	3	4	5	6	7	8	
										mgm.
Test	0.12	0.12	0.13	0.13	0.14	0.14	0.15	0.16	0.17	4.26
Heated control	0.11	0.11	0.11	0.10	0.11	0.12	0.12	0.11	0.12	4.8

The figures are given in milligrams of nitrogen per cubic centimeter

These figures indicate that there is present in tubercle bacilli an enzyme capable of decomposing the nitrogenous constituents of the bacillary bodies in alkaline solution.

Experiment III. By the amino acid α nitrogen liberated in alkaline solution. The above experiment (II) was repeated in all details except that the solution was freed from coagulable proteins by means of fifteen volumes of methyl alcohol, the alcohol evaporated off from the filtrate, the residue taken up by 2 cc. distilled water and this tested quantitatively for amino acid α nitrogen content. The following results were obtained, the figures being given in milligrams amino acids using asparagin as the standard.

	DAYS							
	0	1	2	3	5	7	10	30
Test	0.05	0.06	0.06	0.07	0.08	0.09	0.12	0.14
Heated control	0.06	0.06	0.07	0.05	0.05	0.05	0.06	0.05

This experiment indicates that there is an enzyme in tubercle bacilli capable of splitting off into simple form the amino acid building stones of the bacillary bodies.

Summary. The evidence obtained as to the action of the enzymes of the tubercle bacilli (autolysate) upon casein in alkaline solution and upon the bacillary bodies themselves in alkaline solution seem to indicate that this micro-organism possesses a proteolytic enzyme resembling trypsin in action.

Series II. Proteolytic enzyme acting in acid solution (pepsin-like enzyme)

Experiment I. By its presence in the autolysate. The nephelometric method in which edestin was used was not found to be serviceable for this purpose since physiological salt solution partially precipitated the edestin, and if distilled water was used as a solvent and for preparing the autolysate substances producing a turbidity were obtained from the tubercle bacilli.

Experiment II. By the non-coagulable nitrogen liberated in acid solution. The differences obtained in the non-coagulable nitrogen figures by the Folin micro-method, colorimetrically, were not sufficient to warrant drawing any conclusions.

Experiment III. By the amino acids liberated in acid solution. When using suspensions of bacilli (2 cc. bacillary sediment), killed by toluene (2 cc.) and chloroform (1 cc.) the solution being made 0.2 per cent acid with hydrochloric acid and tested at definite intervals as in series I, experiment III, for amino acid α nitrogen liberated, the results obtained were as follows:

	DAYS							
	0	1	2	3	5	7	10	30
Test	0.04	0.05	0.07	0.07	0.07	0.08	0.08	0.08
Heated control	0.05	0.06	0.05	0.06	0.05		0.05	0.04

Summary. Tubercle bacilli possess a pepsin-like enzyme which, though feeble in action, is capable of liberating amino acid α nitrogen from the bacillary bodies in the presence of 0.2 per cent hydrochloric acid.

Series III. Erepsin-like enzymes

Attempts were made to determine the presence of erepsin-like enzymes by using casein in acid solution and testing nephelometrically, as recommended by Kober, by using both the bacillary emulsion and autolysate therefrom, but both attempts failed on account of turbidities produced in the solutions by the bacillary emulsion and autolysate even before adding the precipitant. It was finally decided to test for erepsin-like enzymes by preparing a peptone from Witte's peptone which was completely precipitated by ten volumes of methyl alcohol so that no perceptible trace was dissolved therefrom by means of methyl alcohol, and using as criterion the splitting of this peptone in acid solution, testing for such splitting by means of Harding and MacLean's quantitative amino acid α nitrogen test.

Four cubic centimeters of tubercle bacilli were diluted with sterile physiological salt solution to 10 cc., 2 cc. toluene and 1 cc. chloroform were added, and the mixture, after shaking thoroughly, was placed in the incubator at 37°C. for twenty-four hours, after which the centrifugated supernatant liquid was filtered through a sterile hard filter paper and divided into two equal portions. One portion (5 cc.) was heated thirty minutes at 100°C., while the other portion (5 cc.) remained unheated. To each was added sufficient sterile physiological salt solution to make 10 cc., sufficient hydrochloric acid to make 0.2 per cent, 2 cc. toluene, 1 cc. chloroform and 1 cc. 0.25 per cent pure peptone solution (completely precipitable by ten volumes of methyl alcohol). From each of these tubes a control of 0.5 cc. was withdrawn and to this ten volumes of methyl alcohol was added, the whole centrifugated at 3000 revolutions until perfectly clear and the supernatant clear methyl alcohol analyzed quantitatively for amino acid content. At definite intervals

after incubation 0.5 cc. amounts were withdrawn and also analyzed for methyl alcohol soluble amino acid α nitrogen content. In making the analyses the amount of amino acids was always compared with a fresh solution of asparagin as standard (0.0317 mgm. amino acid α nitrogen as asparagin) and the figures given are in milligram amino acid α nitrogen as derived from this standard.

	DAYS						
	0	1	2	3	5	7	10
Erepsin test	0.015	0.023	0.024	0.024	0.026	0.026	0.032
Heated control	0.019	0.023	0.022	0.022	0.022	0.019	0.021

Summary. The autolysate from tubercle bacilli possesses an enzyme capable, in 0.2 per cent hydrochloric acid solution, of splitting a completely methyl alcohol precipitable peptone prepared from Witte's peptone into simple amino acid α nitrogen compounds, not precipitable by this means.

Series IV. Nuclease (nucleic acid splitting enzyme)

Experiment I. Nephelometrically. To the autolysate (twenty-four hours in incubator under toluene) obtained from a heavy suspension of tubercle bacilli (2 cc. bacillary residue) in sterile physiological salt solution, was added (to a test and heated control) 1 cc. 0.1 per cent nucleic acid making a total of 6 cc., and 2 cc. toluene and 1 cc. chloroform. The nucleic acid was determined quantitatively by the nephelometric method of Kober. In this preliminary test the heated control was used as the standard and the results obtained in percentage as compared to heated control, were; immediately—100 per cent nucleic acid; three days—80 per cent nucleic acid; and six days 77.5 per cent nucleic acid.

This experiment was then repeated more elaborately in that both the heated control and test were compared with a freshly prepared standard of nucleic acid each time. The standard

contained 0.05 cc. of 0.1 per cent yeast nucleic acid in distilled water and was considered as 100 per cent. The total volume in this experiment was 10 cc. instead of 6 cc. as above and to this was added 1 cc. 0.1 per cent freshly prepared yeast nucleic acid solution. The results obtained, in percentage of the standard nucleic acid solution, were as follows:

	DAYS						
	0	1	3	4	5	7	10
Test	74.3*	56.6	56.0	52.8	51.3	49.1	50.5
Heated control	60.3†	59.8	60.0	60.2	60.6	59.7	58.4

* The turbidity produced in the test solution was greater than that accounted for by the nucleic acid added. No explanation has been found for this except the fact that certain substances may have been liberated by the autolysis of the tubercle bacilli which combine with the precipitant to form a turbidity.

† The turbidity of the heated control after the albumin precipitant was added was also greater than that accounted for by the nucleic acid added but less than that of the unheated test.

Summary. The autolysate from tubercle bacilli possesses an enzyme capable of splitting nucleic acid into simpler form.

Experiment II. In an attempt to corroborate the findings of experiment I by amino acid determinations, the following experiment was performed and the results are given for what they may be worth.

Two cubic centimeter amounts of bacillary residue were prepared in four tubes. Sufficient hydrochloric acid was added in one tube to make the final strength 0.1 per cent; to the second tube sufficient hydrochloric acid to make the final strength 0.1 per cent and 3 cc. 0.1 per cent acid caseinate; the third was kept as a neutral control; and to the fourth was added 3 cc. 0.1 per cent nucleic acid. All were diluted to 10 cc. with physiological salt solution, 2 cc. toluene and 1 cc. chloroform was added and the tubes incubated at 37°C. At various intervals definite amounts of the solution were withdrawn and tested for amino acid α nitrogen content.

	INTERVALS					
	0	36 HOURS	4 DAYS	6 DAYS	8 DAYS	11 DAYS
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
1. HCl to 0.1 per cent	0.020	0.026	0.027	0.031	0.039	0.042
2. HCl to 0.1 per cent and 0.1 per cent acid caseinate	0.022	0.039	0.043	0.048	0.051	0.058
3. Neutral control	0.018	0.027	0.036	0.046	0.053	0.065
4. 3 cc. 0.1 per cent nucleic acid	0.024	0.025	0.028	0.034	0.041	0.043

Series V. Urease

The presence of urease in tubercle bacilli was determined by making an emulsion of human bacilli in physiological salt solution, 3 cc. bacillary residue made up to 32 cc. in each of two tubes, and adding 50 mgm. of urea and 5 cc. toluene to a heated control and a test. After withdrawal of a 3 cc. control (filtering through hard filter paper and using 2 cc. for analysis) the tubes were incubated at 37°C., and samples taken out for analyses at various intervals. The amount of urea decomposed was determined by the Folin aeration method. The enzyme action was stopped by the addition of 1 cc. saturated sodium carbonate to 2 cc. of the filtrate. The results obtained are expressed as milligrams of nitrogen in 2 cc. of filtrate.

	PERIOD OF WITHDRAWAL OF SOLUTIONS					
	0	18 HOURS	2 DAYS	3 DAYS	5 DAYS	8 DAYS
Urease test	0.020	0.060	0.100	0.135	0.134	0.132
Heated control	0.015	0.013	0.014	0.016	0.010	0.012

Summary. Tubercle bacilli possess an enzyme capable of decomposing urea.

Series VI. Diastase and Invertase

The presence of diastase and invertase in the tubercle bacillus was tested by determining the amount of reducing sugar formed from starch and sucrose as performed by Meyers and Rose

using the Lewis and Benedict colorimetric picramic acid method. Among four tubes was divided equally 14 cc. of bacillary emulsion (2 cc. bacillary residue in each). Two of the tubes were heated for thirty minutes at 100°C., in a water bath to destroy the enzymes present. To each of the four tubes was added 2 cc. toluene and 1 cc. chloroform and sufficient sterile physiological salt solution to make a total volume of 10 cc. of emulsion. To two of the tubes, one heated and one unheated, was added 5 cc. of 2 per cent sucrose making a concentration of 1 per cent sucrose, and to the other two, one heated and one unheated, 5 cc. of freshly prepared arrowroot starch emulsion made by adding 2 grams of starch in suspension in cold water to about 75 cc. boiling water and diluting to 100 cc. after cooling.

After thorough mixing, the tubes were centrifugated and 1 cc. of the solution drawn off and tested for reducing sugar. The tubes were then incubated at 37°C., and 1 cc. withdrawn and tested at various intervals thereafter.[3] The results are given in the following table.

		TIME OF TEST IN DAYS					
		0	1	3	6	8	10
Starch....	Not heated	Trace (about 0.1 mgm.)	0.2	0.2	0.2	0.2	0.2
	Heated control	Trace (about 0.15 mgm.)	0.15	0.15	0.2	0.2	0.2
Sucrose...	Not heated	Trace (about 0.15 mgm.)	0.15	0.25	0.3	0.3	0.3
	Heated control	Trace (about 0.2 mgm.)	0.2	0.2	0.25	0.25	0.25

Summary. Tubercle bacilli do not possess an enzyme capable of hydrolyzing starch or sucrose in sufficient amount to be demonstrable by the method used.

[3] Control tests of 1 per cent starch and 1 per cent sucrose to which had been added chloroform and toluene revealed no difference from a blank test with the reagents alone. Varying amounts of glucose, 0.5, 0.4, 0.3, 0.2, and 0.1 mgm., in 1 cc. water to which had been added toluene and chloroform also revealed no influence of the antiseptics upon the accuracy of the test.

Series VII. Elastic and connective tissue digesting enzymes.

In order to demonstrate the presence or absence of elastic or connective tissue digesting enzymes in the tubercle bacilli, an indirect method of attack had to be resorted to. In the first place it was necessary to demonstrate just how much digestion was a result of the action of the enzymes upon the proteins already obtained from the bacilli and then using this as control, to determine whether there was really any digestive action upon the elastic or connective tissues. Since the amino acid α nitrogen method of Harding and McLean was the most delicate and accurate method available for the purpose it was used as the index of the digestion taking place during the course of the experiments.

Experiment I. Elastic tissue digesting enzyme. To 10 cc. of emulsified human tubercle bacilli was added 25 cc. sterile 0.9 per cent salt solution, 5 cc. chloroform, and 10 cc. toluene. This was placed in the incubator at 37°C. for twenty-four hours when it was centrifugated and 8 cc. of the supernatant autolysate was drawn off and filtered through a sterile hard filter paper. The filtrate was divided into three equal portions, one was heated for thirty minutes in boiling water to destroy the enzymes and the other two portions were kept unheated. All were now diluted to 5 cc. and to the heated and one of the unheated portions was added 0.5 gram of elastic tissue prepared from lamb lung.[4] A definite amount of the solution (0.5 cc. each time) was withdrawn immediately, one day, three days, five days, seven days, and ten days after incubation at 37°C. and used for determining the amino acid α nitrogen content. Similarly 8 cc. of autolysate were withdrawn at four days and eight days and tested as above for digestive action upon the elastic tissue. The results of the analyses are given in the following table, the

[4] The elastic tissue was prepared by finely grinding lamb's lung and treating it at room temperature, shaking frequently, with a large amount of 5 per cent potassium hydroxide and 10 per cent acetic acid alternately until the potassium hydroxide and acetic acid solutions used remained absolutely colorless and clear after twenty-four hours contact. The elastic tissue thus prepared was washed with water until absolutely free from acetic acid and was kept for use in distilled water with toluene and chloroform as preservatives.

figures being in milligrams of amino acid α nitrogen per 0.5 cc. using asparagin as standard.

AGE OF AUTOLYSATE		DAYS					
		0	1	3	5	7	10
One day	T	0.014	0.013	0.015	0.019	0.020	0.029
	NHC	0.013	0.014	0.015	0.018	0.018	0.028
	HC	0.015	0.013	0.014	0.015	0.015	0.014
Four days	T	0.016	0.024	0.028	0.031	0.035	0.036
	NHC	0.017	0.026	0.027	0.027	0.030	0.035
	HC	0.021	0.021	0.020	0.023	0.022	0.021
Eight days	T	0.017	0.019	0.019	0.021	0.021	0.020
	NHC	0.018	0.021	0.018	0.019	0.019	0.019
	HC	0.020	0.019	0.020	0.021	0.019	0.020

T, Test, containing autolysate and elastic tissue.
NHC, Non-heated control, containing autolysate alone.
HC, Heated control, containing autolysate (heated) and elastic tissue.

Experiment II. Connective tissue disintegrating enzyme. This experiment was done exactly as in Experiment I with the exception that the 0.5 gram elastic tissue was replaced by 0.5 gram connective tissue prepared from the capsule of large tubercles.[5] The results of analyses given as the amount of amino acid α nitrogen in milligrams per 0.5 cc. of solution are tabulated in the following table.

[5] In order to prepare the connective tissue for this experiment rabbits were injected subcutaneously in the back with fat free tubercle bacilli of which 0.05 gram was used for each site of injection. In order to make a uniform suspension a 10 per cent starch paste was prepared with sterile boiling water and to every 5 cc. was added 0.05 gram tubercle bacilli. The bacilli were well mixed with the starch paste by stirring and the entire amount injected subcutaneously while warm by means of a Murphy glycerine syringe or other pressure syringe. After two to three months a large firm tubercle had formed at the site of injection and was removed. After separating all muscle, blood, and caseous material from the thick connective tissue capsule of the tubercle, the latter was well washed with physiological salt solution, then ground up fine in a meat grinder and washed 12 times with salt solution or until no more turbidity appeared in the salt solution upon vigorous shaking. The connective tissue was then heated for one-half hour at 90°C. to destroy the enzymes and again washed with salt solution. The connective tissue thus prepared was preserved for use in salt solution with chloroform and toluene.

AGE OF AUTOLYSATE		DAYS 0	1	3	5	7	10
One day	T	0.012	0.016	0.015	0.014	0.019	0.022
	NHC	0.012	0.011	0.013	0.019	0.021	0.021
	HC	0.011	0.012	0.013	0.012	0.013	0.013
Four days	T	0.019	0.021	0.021	0.024	0.024	0.031
	NHC	0.021	0.020	0.022	0.027	0.028	0.029
	HC	0.020	0.020	0.019	0.019	0.021	0.019
Eight days	T	0.016	0.017	0.017	0.019	0.019	0.019
	NHC	0.019	0.020	0.023	0.024	0.025	0.024
	HC	0.018	0.018	0.019	0.018	0.019	0.019

T, Test, containing autolysate and tubercle connective tissue.
NHC, Non-heated control, containing autolysate alone.
HC, Heated control, containing autolysate (heated) and connective tissue.

Summary. As a result of the above experiments it may be stated that evidence of the presence of a connective tissue, or elastic tissue, disintegrating enzyme in the tubercle bacillus was not obtained, at least by the methods used for this purpose.

COMPLETE SUMMARY

1. Tubercle bacilli of both the human and bovine varieties possess autolytic enzymes, as indicated by the non-coagulable nitrogen and amino acid α nitrogen liberated at incubator temperature after the bacilli have been killed by toluene and chloroform.

2. The bacilli themselves, or autolysates therefrom, also possess a trypsin-like enzyme capable of splitting proteins in alkaline solution, an erepsin-like enzyme capable of decomposing peptone in acid solution, a weak pepsin-like enzyme capable of splitting proteins in acid solution, a nuclease capable of splitting nucleic acid and a urease capable of decomposing urea.

3. The tubercle bacilli, or autolysates therefrom, do not possess enzymes capable of hydrolyzing starch or inverting sucrose, demonstrable by the delicate Lewis and Benedict picramic acid method.

4. Autolysates from tubercle bacilli do not possess enzymes capable of digesting elastic tissue prepared from lamb lung, or connective tissue prepared from tubercles, at least, as indicated by the methods used for demonstrating these enzymes.

REFERENCES

Bock, J. C. and Benedict, S. R. 1915 J. Biol. Chem. **20**, 47.
Eijkman, C. 1901 Centrabl. f. Bakteriol. I Abt. **29**, 841.
1913 Centralbl. f. Bacteriol. I Abt. **35**, 1.
Folin, O. and Farmer, C. J. 1912 J. Biol. Chem. **11**, 493.
Folin, O. and Macallum, A. B. 1912 Ibid. **11**, 523.
Fontes, A. 1911 Memorias do Instit. Oswaldo Cruz. **3**, 196. Abstract, 1912. Centralbl. f. Bakteriol. I Abt. Ref. **55**, 14.
Gosio 1905 Zeitschr. f. Hyg. U. Infectionskh. **51**, 65.
Graves, S. S. and Kober, P. A. 1914 J. Am. Chem. Soc. **36**, 751.
Greenwald, I. 1915 J. Biol. Chem. **21**, 61.
Harding, V. J. and MacLean, R. M. 1915 J. Biol. Chem. **20**, 217.
Harding, V. J. and MacLean, R. M. 1916 J. Biol. Chem. **24**, 503.
Jobling, J. W. and Peterson, W. 1914 J. Exper. M. **19**, 251.
Kendall, A. I., Day, A. A. and Walker, A. W. 1914a J. Infect. Dis. **15**, 433.
Kendall, A. I., Day, A. A. and Walker, A. W. 1914b J. Infect. Dis. **15**, 439.
Kendall, A. I., Day, A. A. and Walker, A. W. 1914c J. Infect. Dis. **15**, 443.
Kendall, A. I., Day, A. A. and Walker, A. W. 1914d J. Infect. Dis. **15**, 451.
Kober, P. A. 1913 J. Am. Chem. Soc. **35**, 290.
Kober, P. A. and Graves, S. S. 1914 J. Am. Chem. Soc. **36**, 1304.
Lewis, R. C. and Benedict, S. R. 1915 J. Biol. Chem. **20**, 61.
Myers, V. C. 1916 Science **44**, 215.
Myers, V. C. and Rose, A. R. 1916 Science **44**, 215.
Opie, E. L. and Barker, B. 1908 J. Exper. M. **10**, 645.
Opie, E. L. and Barker, B. 1909 J. Exper. M. **11**, 686.
Wells, H. G. and Corper, H. J. 1912 J. Infect. Dis. **11**, 388.

SOME CHARACTERS WHICH DIFFERENTIATE THE LACTIC-ACID STREPTOCOCCUS FROM STREPTOCOCCI OF THE PYOGENES TYPE OCCURRING IN MILK

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INTRODUCTION

Much confusion exists in bacteriological literature concerning the identity of the lactic-acid streptococcus and its relation to the other chain-forming cocci which occur in milk. It is generally considered that the true lactic-acid bacteria (variously known as *Bact. lactis-acidi*, *Streptococcus lacticus*, *B. lactici-acidi*, *Bacterium* or *Streptococcus guntheri*, etc.) comprise a definite and rather well defined group of organisms. But no sharp points of distinction have been established between these organisms and other streptococci, notably the pyogenic streptococci, which are usually, if not always, to be found in market milk.

The descriptions given for the lactic-acid streptococcus in the older publications on systematic bacteriology, such as the books by Migula (1900) and Chester (1901), could be applied equally well to the pathogenic streptococci. The same confusion is found in the more recent literature. Weigmann (1911) includes the streptococcus of bovine mammitis in the same group as the true lactic-acid organism. Ernst (1914) says that there is no known method by which the lactic-acid streptococcus and the mammitis streptococcus can be differentiated. Jordan (1915) states that "the milk streptococcus in all its properties is extraordinarily like *Streptococcus pyogenes*." Heinemann (1906) who has extensively studied the lactic-acid bacteria, concludes that

"*Streptococcus lacticus* agrees in morphological, cultural and coagulative properties with pathogenic, fecal and sewage streptococci."

Certain morphological characters are emphasized by some workers as differentiating the true lactic-acid organisms from other streptococci. It is pointed out that the cells of the milk streptococcus are usually elongated and also that the characteristic grouping is in pairs and short chains rather than in long chains such as are usually found in the true streptococci. These points cannot be used as sufficient ground for differentiation since it is well known that many cultures of the lactic-acid bacteria appear as perfect cocci, and the formation of the long chains is not uncommon. Further, the streptococci from pathological sources are frequently of the short chain type. Chain formation, cell size and cell shape are so greatly influenced by the nature of the nutrient medium that distinctions based upon them are bound to be of doubtful value.

Hastings (1911) has called attention to the fact that the lactic-acid organism when grown in litmus milk causes a complete reduction of the litmus previous to the curdling of the milk. This is not true of the streptococci in general with which, as a rule, the action on litmus is more gradual and not so complete. This character has been used by Hastings and his associates to differentiate the true lactic-acid bacteria from other streptococci which occur in milk and cheese. Unless, however, this test can be correlated with some other points of difference, we would not be justified in accepting it alone as giving a firm basis upon which to separate the different groups of milk streptococci.

From the study of a large collection of streptococci isolated from milk, infected udders, saliva and feces of cows, Rogers and Dahlberg (1914) were able to show that the streptococci of milk resemble those which are associated with mammitis in cattle, and that the types occurring in the cows' mouths and feces are present only in small numbers and are probably relatively unimportant in milk. They further pointed out that the streptococci from infected udders had the same physiological characteristics as the well known *Streptococcus pyogenes*. It has

recently been demonstrated (Sherman and Hastings, 1914; Evans, 1916) that a large proportion of healthy milch cows harbor streptococci within their udders which are morphologically and culturally identical with the typical *Strept. pyogenes* of mammitis and other pathological conditions. Hence such organisms are probably always present in milk produced from any considerable number of animals.

From all of these facts it would appear that there are two main groups of streptococci in milk, the true lactic-acid organisms and streptococci of the pyogenes type whose chief source is the udder. The lines of cleavage between these groups should certainly be established, and it was with the object in view of finding points of differential value that the present study was undertaken.

SELECTION OF CULTURES

The ordinary way in which to attack a problem of this kind would be to isolate a large number of miscellaneous chain-forming cocci from various grades of market milk and milk products and to subject them to a detailed study with the intention of separating them in that way into their natural divisions. Our manner of approach has been different in that the cultures were obtained in such a way as would tend to limit the collection to organisms typical of the groups under consideration, and not to include a great number of closely related types which would complicate the study and make more difficult their separation into natural groups. Since the true lactic-acid organism has not been defined so as to enable it to be distinguished from other closely related bacteria, it is obviously impossible to tell just which are and which are not typical lactic streptococci. It would seem, however, that the organisms which take an active part in the natural souring of dinorary market milk could be properly designated as true lactic-acid bacteria. Proceeding on this assumption, cultures were isolated by the following method:

Ten samples of raw milk and cream were obtained from the receiving vat of the Pennsylvania State College Creamery on as many different days. These samples were of the mixed prod-

uct from a large number of different dairy farms and so might well be considered as representative of market milk or cream of ordinary quality. After collection the milks were allowed to stand at laboratory temperature until the acidity began to rise. When the acidity reached about 0.2 per cent lactic-acid the samples were plated on lactose agar in dilutions of 1/10,000,000 cc. The use of such a high dilution should result in the isolation of the organisms which are taking the most active part in the acid fermentation. The plates were incubated at 37°C. for two days, after which small, dense colonies surrounded by a hazy precipitate[1] were fished off into sterile milk tubes. Five colonies were selected from each sample making a total of fifty cultures of this type. Without exception the bacteria selected in this way produced an acid fermentation with the formation of a smooth homogeneous curd. No consideration was given to morphology in the selection of these cultures. The cultures of this group were numbered from 1 to 50 inclusive, and after being tested for purity, were added to the collection.

Cultures representing the pyogenic type of streptococci were obtained as follows: Samples were taken from the individual cows of the Pennsylvania State College dairy herd by drawing the milk directly from the udder into sterile flasks. Samples of the mixed milk from the College herd were also obtained. This milk is of a very high grade, being produced under the best of sanitary conditions, and its flora is consequently made up chiefly of the types of bacteria which come from the udder.

These milks were plated in dilutions[2] of 1/100 on lactose agar and incubated at 37°C. for two days. Colonies similar to those of the lactic-acid organisms mentioned above were transferred to glucose broth. Cultures which formed chains in broth were

[1] Bacteriologists engaged in milk studies are familiar with the precipitate formed around the colonies of the lactic-acid bacteria when grown on agar made with Witte's peptone and containing a fermentable sugar. When grown on media made with some of the American brands of peptone this characteristic is not seen. Witte's peptone was used in the preparation of the agar from which these cultures were isolated.

[2] In the case of cow 459, which had garget, the milk was plated in a dilution of 1/1,000,000 cc.

saved for study. The fifty cultures selected came from the milks of seven individual cows and from the mixed herd milk as follows:

Culture Numbers	*From Cow*
51 to 63	663
54 to 60	337
61 to 66	459
67 to 72	631
73 to 77	734
78 to 83	620
84 to 90	624
91 to 100 mixed herd milk	

All of the animals from which these samples were obtained were in a healthy condition, except cow 459, which was suffering from a case of mammitis.

MORPHOLOGY

Microscopic examinations were made of all of the 100 cultures on agar, broth and bile. Although no hard and fast rules can be given, it may be said from this study that on agar and broth the group of organisms numbered from 1 to 50 showed a greater tendency to form elongated cells than did the others. Also the typical grouping, that is the grouping of the majority of cells, was usually in pairs rather than in definite chains. But chain formation was common in these cultures, and in some of them it was the predominating grouping. The grouping in pairs was frequent among the cultures numbered from 51 to 100, but in all of these the typical arrangement was in chains.

The results obtained from growth on lactose-peptone-bile were surprising in that the lactic-acid cultures made a much more luxuriant growth than did the udder streptococci. On this medium the lactic organisms grew in long chains and appeared as typical streptococci in every way. In only one of these cultures on bile were as many cells arranged in pairs as were present in chains. This is of special interest in view of the fact that many health laboratories use bile as a presumptive test for the presence of undesirable streptococci in milk. Kinyoun and Dieter (1912) consider that the formation of chains in bile

inoculated with milk is evidence of fecal contamination, while Rogers, Clark and Evans (1916) believe from their results that it is indicative of infected udders in some of the milk producing animals. In our tests the streptococci from the udder grew more feebly in bile than did the lactic-acid streptococci, and, in most cases, could be found only with difficulty in microscopic preparations. When examined after two days incubation at 37°C. bacteria were observed in only 17 of the 50 cultures. Those seen were all in typical chain formation.

In table 1 is presented a summary of the results obtained in this study. The examinations of broth and agar cultures were made after incubation at 37°C. for one day. The growth ex-

TABLE 1

Chain formation on agar, broth and bile

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Chains on lactose agar	58	100
Chains on glucose broth	50	100
Chains on lactose-peptone-bile	100	100
Chains predominating on broth	24	100
Chains predominating on bile	98	100

amined from agar was taken from the sloped surface, not from the water of condensation. Chain formation was not recorded as positive unless definite chains of ten or more cells in length were seen.

These data indicate that some distinction may be drawn between these two groups of streptococci based upon cell grouping. For example, all of the cultures numbered from 51 to 100 showed chains on agar and broth, while this was true of only about one-half of the organisms from the other group. If consideration is taken of the grouping of the majority of cells a greater difference is seen. On broth in all of the streptococci of the udder type the predominating arrangement was in chains whereas this was the case in only about one-fourth of the other cultures.

Although it might appear that chain formation is of some value in distinguishing the lactic-acid bacteria from other streptococci, we do not wish to make that claim. Such a differentiation would at best be a doubtful one. The method used in collecting cultures for this work was such as would tend to introduce an error in this phase of the study; only cultures which showed typical chain arrangement were selected as representing the udder-type of streptococcus, while no attention was paid to morphology in selecting the lactic-acid type. As was noted before, chain formation is not a very constant character. In this study there was apparently no correllation between the lactic cultures which grew in chains on broth and those which

TABLE 2

Action on milk

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Milk curdled	100	100
Milk curdled in twenty-four hours	94	20
0.75 per cent lactic acid in milk	80	8

did so on agar, and it is doubtful if they would show much constancy in this respect on the same medium.

ACTION ON MILK

The amount of acid produced in milk, the presence of coagulation and the time required for curdling were noted. All of the cultures produced sufficient acid to cause the coagulation of milk when tested at 37°C. As may be seen from table 2, the organisms of the first group (cultures 1 to 50) showed a more prompt clotting of milk than did those of the second group. At 37°C. all but 3 in the first group curdled milk within twenty-four hours, while only 10 of the other group acted so promptly.

The maximum acidity produced in milk was tested by incubating the cultures for ten days at 35°C. The acidity developed

at this temperature would not be so high as if a lower incubation temperature were used, but for comparing the two classes of organisms it should serve the purpose. By this test it was found that the lactic-acid type of streptococci (cultures 1 to 50) as a group produced considerably larger amounts of acid in milk than the udder cultures. If an arbitrary standard of 0.75 per cent lactic acid is taken (table 2) it will be seen that the two groups are divided quite well by the acid producing powers of

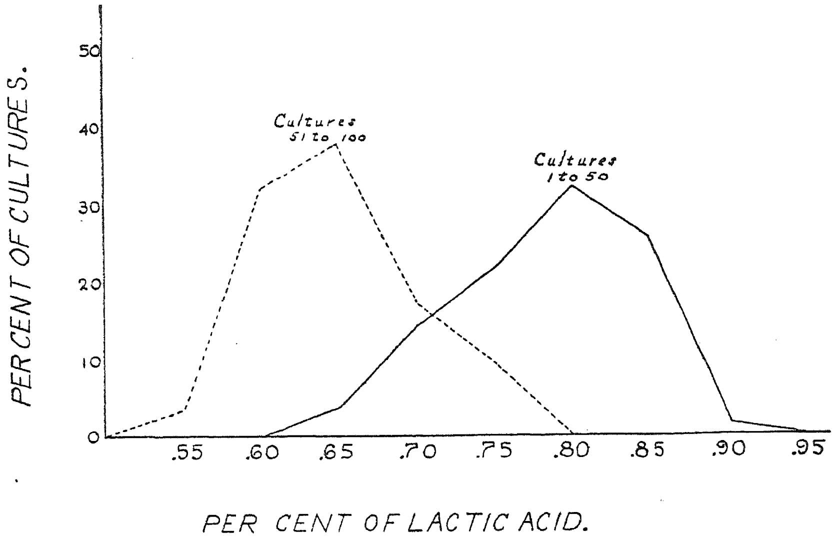

FIG. 1. FREQUENCY CURVE SHOWING ACID PRODUCTION IN MILK

their members. The difference in acid production is better shown by the frequency curves presented in figure 1.

These curves show a marked difference in the modes of the two groups as to acid production in milk. The same thing is true with respect to acid formation in lactose-peptone-bile. As was noted before, the lactic cultures appeared to grow more vigorously in bile than did the other streptococci, and the difference in the amounts of acid formed by the two types in this medium gave further evidence of that fact.

Although these results would appear to indicate that the true lactic-acid organisms as a class are capable of enduring a higher degree of acidity and of producing a more rapid coagulation of milk than the pyogenic streptococci, it is desirable to qualify this conclusion. The manner of securing the lactic streptococci was such as would tend to result in the collection of strains which were especially vigorous in their growth and action on milk. As is well known, the amount of acid produced

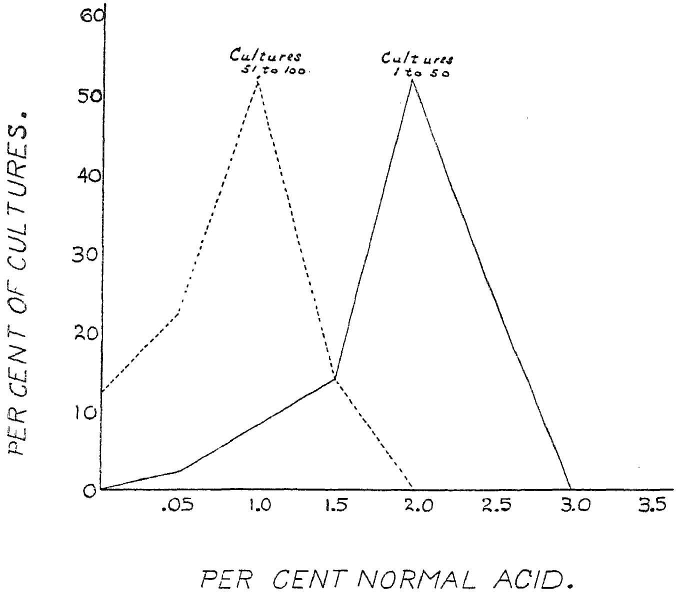

FIG. 2. FREQUENCY CURVE SHOWING ACID PRODUCTION IN LACTOSE-PEPTONE-BILE

in milk is not a very stable character and may be greatly altered by various factors such as, for example, growth on laboratory media. However, the degree of titratable acidity developed is probably of some differential value and it is not unlikely that determinations of actual hydrogen-ion concentrations, such as were made by Clark and Lubs (1915) with the colon-aerogenes bacteria and by Ayers (1915) with pathogenic and non-pathogenic streptococci, would reveal a fundamental difference between these two groups.

FERMENTATIVE CHARACTERISTICS

The value of fermentation tests with carbohydrates and related compounds in systematic bacteriology has become well established. The fermentative characteristics have been made use of especially in studies on streptococci, and by their means this very evasive group has been subdivided into a number of fairly well defined types. These tests have not been used, successfully at least, to distinguish between streptococci of the pyogenic and lactic-acid types.

In this study glucose, galactose, levulose, maltose, lactose, sucrose, raffinose, dextrin, inulin, starch, glycerine, mannit and salicin were used. The medium used for the fermentation tests had the following composition:

	per cent
Beef extract	0.3
Peptone	1.0
Dibasic potassium phosphate	0.5
Test substance	1.0

In the tests made with glucose, galactose, levulose and maltose the dibasic potassium phosphate was omitted.

In making the tests the cultures were incubated at 33°C. and then titrated against $\frac{N}{20}$ NaOH, with phenolphthalein as indicator, and the results expressed as per cent of normal acid. An increase above the control tube of 1 per cent normal acid was regarded as a positive test for fermentation. The sugars were incubated seven days and the other compounds two weeks before titration. In cases in which less than 25 per cent of the organisms were either positive or negative the minority cultures were retested. The results of this study are summarized in table 3.

A review of these data shows that the fermentative properties of the two groups are, generally speaking, quite similar. Although resembling each other on the whole, a very noticeable break in the similarity is found in the case of sucrose. It is seen that 76 per cent of the udder organisms attacked this substance as against only 6 per cent of the other group. The fermentative powers of the cultures are presented graphically in figure 3.

In this graph only the test substances are given which showed a difference of at least 5 per cent between the two groups.

From this it is evident that aside from sucrose none of the substances show sufficient difference to be of differential value. Exception might be taken to this statement in the case of maltose, in which all of the udder streptococci reacted positively while about one-fourth of the cultures of the other group were negative, but this could hardly be considered of great value for purposes of identification. Though none of the udder cultures of this collection fermented mannit, that fact is probably of no

TABLE 3

Fermentation of test substances

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Glucose	100	100
Galactose	84	100
Levulose	94	100
Maltose	76	100
Lactose	100	96
Sucrose	6	76
Raffinose	0	0
Dextrin	8	18
Inulin	0	0
Starch	0	2
Glycerine	0	8
Mannit	28	0
Salicin	28	16

significance from the point of view of classification because it is well known that the ability to attack this substance is not uncommon among streptococci of the pyogenes type. In the work of Rogers and Dahlberg (1914) about one-fourth of the udder streptococci (from infected udders) fermented mannit, and this character was also common among cultures from other sources.

Though having no relation to the purpose of this paper, it is interesting to note that among the udder organisms all of the six cultures from one cow (no. 631) fermented salicin while only

two cultures of the remaining forty-four had that property. Of greater interest perhaps is the fact that five of the six cultures taken from cow 459, the only animal which had an infected udder, fermented dextrin. Whether this was merely a coin-

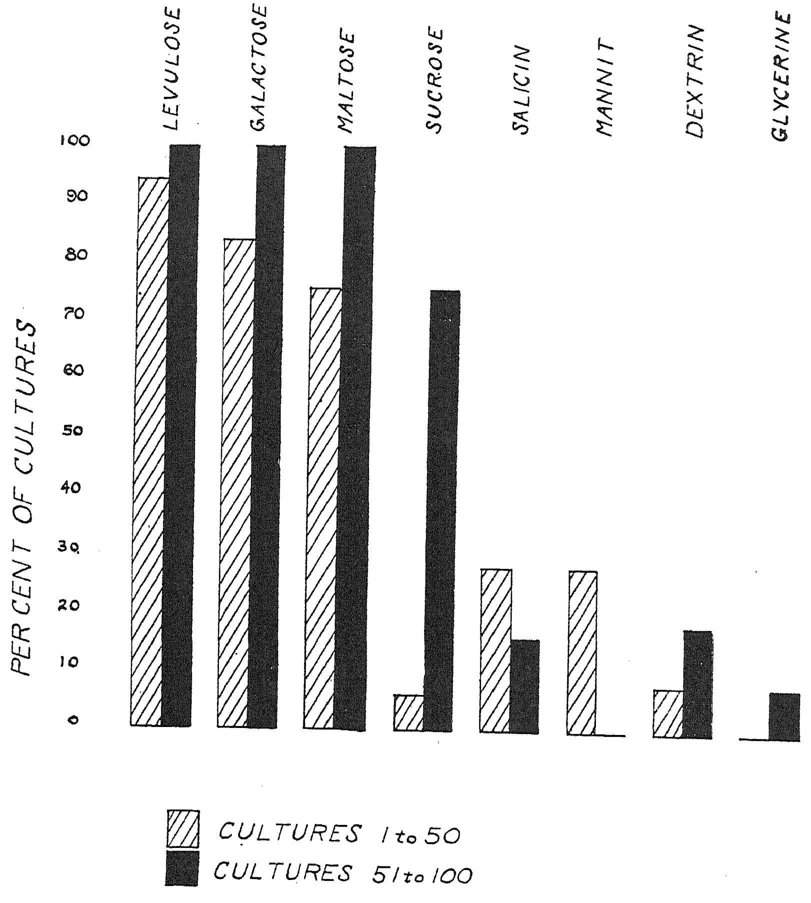

Fig. 3. Graph Showing Differences in Fermentative Properties of Cultures Studied

cidence or whether there exists a relation between the ability to attack dextrin and pathogenicity would require further study to answer. Two of the udder cultures failed to ferment lactose. Lactose negative streptococci have been shown by Andrewes

and Horder (1906) and by Winslow and Palmer (1910) to be numerous in the intestinal contents of the horse. In the present case, the two cultures in question not only caused an acid fermentation in milk, but produced such a vigorous one as to coagulate the casein. It seems, therefore, that these organisms were able to ferment lactose under favorable conditions but could not attack it for some reason when growing in the nutrient broth employed for the tests. A similar idiosyncrasy is noted in a few of the cultures reported by Rogers and Dahlberg (1914).

If it is conceded that the organisms of this collection numbered from 1 to 50 are representative of the group of true lactic-acid streptococci some significant points are brought out. It is generally stated in bacteriological texts[3] that the lactic organism ferments sucrose, while the statement is also found that mannit is one of the substances attacked, but according to our results the group is typically negative with both of these substances though some strains can ferment them. The typical *Strept. lacticus*, it would appear, ferments glucose, galactose, levulose, maltose and lactose. Of the cultures which failed to ferment either levulose or galactose none attacked maltose. Among the fifty cultures used there were fourteen which acted upon mannit and a similar number which fermented salicin. Nine of these attacked both compounds, thus showing quite a marked correlation between the fermentation of mannit and salicin. In most cases the cultures which fermented sucrose or dextrin also attacked both mannit and salicin.

TEMPERATURE RELATIONS

It is generally thought that the true lactic-acid streptococcus grows better at a lower temperature than the optimum for most pathogenic bacteria. Although it has not been demonstrated with any considerable number of cultures, the statement is usually made that the lactic organism has an optimum temperature of from 30°C. to 35°C. Stowell, Hilliard and Schlesinger

[3] See Weigmann's Mikologie der Milch; Marshall's Microbiology and Buchanan's Household Bacteriology.

(1913) have shown that streptococci from milk are, as a rule, more active at 20°C. than those isolated from the human throat.

Experiments at low temperatures of incubation were conducted, by inoculating litmus milk with the cultures and incubating at the desired temperature. Growth was determined by the presence or absence of visible changes in litmus milk. At 10°C. the two groups were differentiated perfectly; all of the cultures numbered from 1 to 50, which were supposed to represent the *Strept. lacticus* type, grew, while none of the group representing the *Strept. pyogenes* type did. The latter showed no change in the litmus milk after six weeks, but when put in a 37°C. incubator at the end of this period the tubes all turned acid, thus indicating that the cultures were alive but their

TABLE 4

Temperature relations

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Growth at 10°C.	100	0
Growth at 43°C.	6	82

growth had been inhibited. The lactic streptococci all showed visible signs of growth within one week at 10°C.

Tests similar to the above were also made with high temperatures of incubation. At 43°C. was found a temperature which separated the two groups quite well, but the separation was not so perfect as at 10°C. In this case it was the pyogenic type which grew and the lactic type which failed to grow.

REDUCTION OF DYES

The use of stains as an aid in the identification of bacterial groups is not uncommon. Some familiar examples are the employment of neutral red, fuchsin and brilliant green for differentiating members of the colon-typhoid group of organisms. The reduction of neutral red was one of the characters advocated by Gordon (1905) as being of value in the separation of strep-

tococci. Rogers and Davis (1912) considered the reduction of this compound of differential value in their study of the lactic-acid bacteria of milk. As was noted earlier, Hastings (1911) has used the action on litmus in milk to distinguish the lactic-acid organism from other streptococci.

A preliminary survey of the various stains indicated that methylene blue, neutral red, litmus and indigo carmine might be of service in the present work. According to Fred (1912), bacteria, at least those types common in milk, reduce stains more actively in a milk medium than in broth, and a few tests made at the beginning of this study verified this conclusion. Tests were made by adding the dye to sterilized whole milk, the advantage of unskimmed milk being that the fat forms a layer over the surface which excludes the air quite effectively and thus reduction is not hindered. The litmus milk was prepared in the ordinary way by adding sufficient litmus solution to the milk to give a rather dark lavender color and then sterilizing. The other dyes were made as follows:

Methylene blue

Medicinal methylene blue	0.5 gram
Distilled water	1000 cc.

Indigo carmine

Indigo carmine (Kahlbaum's)	1.0 gram
Distilled water	1000 cc.

Neutral red

Neutral red (Grübler's)	0.1 gram
Distilled water	1000 cc.

The stain solutions and milk were sterilized separately and then mixed in the proportion of 1 cc. of stain to 10 cc. of milk.

In making the tests twenty-four hours old cultures of the organisms in milk were used to inoculate from, and the stain culture so prepared was incubated at 37°C. Observations were then made on three points, (1) reduction of stain, (2) time required to reduce, and (3) whether reduction was before or after curdling of milk. When no reduction was evident the cultures were allowed to remain six days before final examination was made.

As may be seen from table 5 the reduction of dyes proved to be an efficient test to differentiate between these two groups of bacteria. Methylene blue differentiated the groups perfectly, all of the true lactics causing a complete reduction of the stain within twenty-four hours and previous to curdling, while the udder streptococci failed entirely to cause reduction. After six days the tubes containing the latter class were not curdled and there was no evidence that growth had taken place. Some of these cultures were transferred to plain milk tubes but no growth occurred, thus indicating that not only were they unable

TABLE 5

Reduction of dyes

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Methylene blue	100	0
Methylene blue (within twenty-four hours)	100	0
Methylene blue (before curdling)	100	0
Litmus	100	100*
Litmus (within twenty-four hours)	100	0
Litmus (before curdling)	100	0
Indigo carmine	100	100*
Indigo carmine (within twenty-four hours)	100	0
Indigo carmine (before curdling)	100	0
Neutral red	92	0
Neutral red (within twenty-four hours)	84	0
Neutral red (before curdling)	4	0

* Reduction not complete.

to reduce methylene blue, but that, in the concentration used, it had entirely inhibited growth and led to their destruction. The longest time required for any of the lactic-acid streptococci to reduce was ten hours, while a large majority caused a complete decolorization within eight hours after inoculation.

The actions on litmus and indigo carmine were apparently identical. In both cases the lactic-acid streptococci caused a prompt reduction; with litmus the longest time taken was eleven hours, while with indigo carmine thirteen and one-half hours was the longest time required. The udder cultures, on

the other hand, caused a reduction but it did not take place until after two or more days and then it was never absolutely complete. The important distinction, however, is that the reduction in the lactic cultures took place previous to curdling, whereas the pyogenic streptococci caused no reduction until after coagulation. This we believe represents a fundamental physiological difference between the two groups and it is to be considered, therefore, of value as a differential test. Since the litmus, indigo carmine, methylene blue and low temperature tests are all perfectly correlated, it would appear that the method of distinguishing between lactic-acid organisms and other milk streptococci based upon whether they cause reduction in litmus milk before curdling is sound.

Neutral red, which has been used considerably in studying streptococci, proved the least valuable of the four stains employed. Though the two groups were separated quite well, the distinction based upon this dye was not a perfect one. The lactic-acid organism did not cause as prompt reduction with neutral red as with the other stains, it requiring from twelve to fifty hours to decolorize this compound; and the reduction took place in most cases after curdling. Only four of the lactic cultures failed to reduce while all of the other streptococci gave negative reactions.

DISCUSSION

From the data which have been presented, it seems justifiable to conclude that the organisms studied represent two classes of streptococci; cultures numbered 1 to 50 the true lactic-acid streptococcus, and the other group (cultures 51 to 100) the pyogenic type. A review of the tests made will show a number of characters which differentiate the cultures into those two main types. The diagram given in figure 4 shows very clearly some of the fundamental differences between these groups. In this graph are presented only those tests which appeared to be of considerable differential value, those showing only slight differences between the two types being omitted.

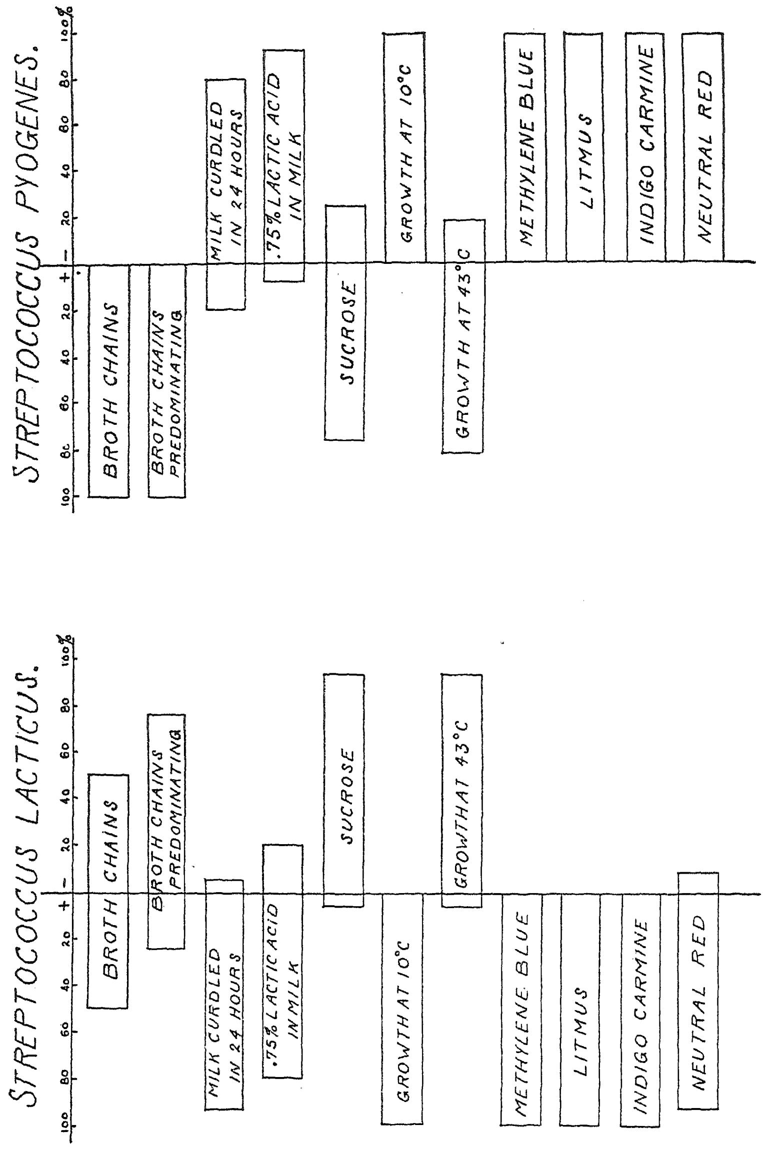

FIG. 4. DIAGRAM SHOWING DIFFERENCES BETWEEN THE LACTIC-ACID AND PYOGENIC STREPTOCOCCI

These tests, it is thought, are of sufficient value to make possible the separation of these two groups of streptococci, but it would be advantageous further to limit the number of tests and to establish the most constant ones. The differences in the groups relative to chain formation and action on milk are, in all probability, significant, but for reasons given earlier in this paper, it is not desirable to emphasize them greatly. The remaining characteristics, however, we believe to be stable ones —as stability occurs in the physiological characters of bacteria. Among the dyes neutral red did not give an absolute differentiation of the cultures studied and so might be eliminated in favor of methylene blue. As the action on indigo carmine was identical in all cases with that on litmus, it also might be discarded. The five remaining tests we wish especially to recommend as furnishing a ready and simple means of separating the milk streptococci. Growth at 10°C., reduction of methylene blue in milk, and the reduction of litmus (or indigo carmine) in milk previous to curdling are characters which have divided perfectly, in our study, the two types; *Strept. lacticus* having in all cases reacted positively while the *Strept. pyogenes* cultures, without exception, failed so to act. Absolute differentiation was not obtained with the other two tests—growth at 43°C. and the fermentation of sucrose—but the *Strept. pyogenes* type, in the great majority of cases, gave positive reactions whereas the lactic-acid streptococci in both instances were usually negative.

Although the object of this work was only to establish points of difference between the two groups of organisms, it should, aside from its main purpose, be of value in helping to define more clearly the characteristics of the *Strept. lacticus* (*Bact. lactis-acidi*) group of bacteria. As was pointed out previously, our results do not substantiate some of the generally accepted ideas concerning its fermentative properties. The facts established with reference to its temperature requirements and reducing ability, should so identify the true lactic-acid organism as to enable more reliable work than has been possible in the past concerning morphology, physiology, natural habitat and pathogenicity.

SUMMARY

A study was made of 100 cultures of organisms isolated from milk. The collection was so made that 50 of these cultures, it is believed, represented the true lactic-acid streptococcus and the other 50, streptococci of the pyogenes type.

Morphological observations were made from agar, broth and bile. The tendency to form chains on agar and on broth was not so marked among the cultures of the *Strept. lacticus* group as among the organisms of the *Strept. pyogenes* type. On lactose-peptone-bile, however, the *Strept. lacticus* cultures grew readily and formed long, typical streptococcic chains.

Among the cultures studied, the representatives of the *Strept. lacticus* type as a class had a more vigorous action on milk than did the other streptococci; coagulation was usually more prompt and larger amounts of acid were formed.

The fermentative characteristics of the two groups were quite similar with all of the substances used except sucrose. This compound was attacked by 38 of the 50 cultures of the pyogenic streptococci, while all but three of the lactic-acid streptococci failed to ferment it.

At 10°C. all cultures of the lactic-acid bacteria grew while none of the cultures of the *Strept. pyogenes* type were able to develop at this temperature. At 43°C. only 3 of the lactic organisms grew, whereas the pyogenic streptococci developed in 84 per cent of the cases.

The reduction of stains proved a valuable means of distinguishing these groups. With methylene blue all of the lactic streptococci caused reduction, while all of the pyogenic streptococci failed to reduce. Litmus and indigo carmine in milk were completely reduced before curdling by all of the *Strept. lacticus* cultures; the cultures of *Strept. pyogenes* caused no decolorization of these compounds previous to curdling, and the reduction after coagulation was slow and never absolutely complete. Neutral red, though of value, did not give as perfect a differentiation between the two types as did the other dyes.

Differences in chain formation and action on milk are doubtless of some importance, but differentiation based on these characters is probably not to be recommended. The reduction of stains, the fermentation of sucrose and the temperature tests, on the other hand, are believed to represent more constant characters and to offer means by which these two groups of streptococci may be differentiated.

REFERENCES

ANDREWES, F. W. AND HORDER, T. J. 1906 A study of the streptococci pathogenic for man. Lancet, **171**, 708–713.

AYERS, S. H. 1915 Hydrogen-ion concentrations of cultures of streptococci. Meetings of Soc. Am. Bacteriologists, Urbana, Illinois.

CHESTER, F. D. 1901 A manual of determinative bacteriology. New York: Macmillan Company.

CLARK, W. M. AND LUBS, H. A. 1915 The differentiation of bacteria of the colon-aerogenes family by the use of indicators. J. Infect. Dis., **17**, 160–173.

EVANS, ALICE C. 1916 The bacteria of milk freshly drawn from normal udders. J. Infect. Dis., **18**, 437–476.

ERNST, W. 1914 Milk hygiene; translated by Mohler and Eichhorn. Chicago: Alexander Egar.

FRED, E. B. 1912 A study of the quantitative reduction of methylene blue by bacteria found in milk and the use of this stain in determining the keeping quality of milk. Centralbl. f. Bakteriol. (etc.), Abt. 2, **35**, 391–428.

GORDON, M. H. 1905 Report of some characters by which various streptococci and staphylococci may be differentiated and indentified. 33rd Ann. Rpt. Local Government Board (Great Britain), containing report of Medical Officer, 388–430.

HASTINGS, E. G. 1911 *In* Marshall's Microbiology. Philadelphia: P. Blakiston's Sons and Company.

HEINEMANN, P. G. 1906 The significance of streptococci in milk. J. Infect. Dis., **3**, 173–182.

JORDAN, E. O. 1915 General bacteriology. Philadelphia: W. B. Saunders Company.

KINYOUN, J. J. AND DIETER, L. V. 1912 A bacteriological study of the milk supply of Washington, D. C. Am. J. Pub. Health, **2**, 262–274.

MIGULA, W. 1900 System der Bakterien. Jena: Gustav Fischer.

ROGERS, L. A. AND DAVIS, B. J. 1912 Methods of classifying the lactic-acid bacteria. U. S. Dept. Agr., B. A. I. Bull. 154.

ROGERS, L. A. AND DAHLBERG, A. O. 1914 The origin of some of the streptococci found in milk. J. Agric. Research, **1**, 491–511.

Rogers, L. A., Clark, W. M. and Evans, A. C. 1916 Colon bacteria and streptococci and their significance in milk. Am. J. Pub. Health, **6**, 374–380.

Sherman, J. M. and Hastings, E. G. 1914 The presence of streptococci in the milk of normal animals. Meetings Soc. Am. Bacteriologists, Philadelphia, Pa.

Stowell, E. C., Hilliard, C. M. and Schlesinger, M. J. 1913 A statistical study of the streptococci from milk and from the human throat. J. Infect. Dis., **12**, 144–164.

Weigmann, H. 1911 Mikologie der Milch. Berlin: Paul Parey.

Winslow, C.-E. A. and Palmer, G. T. 1910 A comparative study of streptococci from the horse, the cow and man. J. Infect. Dis., **7**, 1–16.

STUDIES IN THE NOMENCLATURE AND CLASSIFICATION OF THE BACTERIA

V. SUBDIVISIONS AND GENERA OF THE SPIRILLACEAE, AND NITROBACTERIACEAE

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Family III. **Spirillaceae** Migula, 1894, p. 237

Synonyms:

Spirillina Perty, 1852, p. 179
Spirillineae Cohn, *in ed.* Rabenhorst, 1865, p. 70
Spirobacteria Cohn, 1872, p. 180
Vibrioneae Trevisan, 1879, p. 139
Spirobacteriaceae Hueppe, 1886, p. 143
Spirilleae De Toni and Trevisan, 1889, p. 1006

Cells elongate, not spherical, bent in the form of a spiral or a segment of a spiral. Usually motile by means of polar flagella. Without sulfur granules or bacteriopurpurin.

Perty (1852) subdivided the *Spirillina* into the genera *Spirillum* and *Spirochaeta*.

Cohn (1872) likewise divided the tribe *Spirobacteria* into *Spirillum* with cells rigid, relatively short, and *Spirochaete*, with cells flexuous and relatively long. In 1875 Cohn described the additional spiral genus *Myconostoc* in which the spiral cells occurred embedded in a mass of gelatin, and included *Vibrio*, *Spirillum*, *Spirochaete* and *Myconostoc* in the algal tribe *Nematogenae*. The genera may be separated by the following key.

Key to genera of spiral Nematogenae Cohn

A. Cells not united by gelatin into families.
  1. Filaments short, sinuous.................................*Vibrio*
  2. Filaments short, spiral, rigid........................*Spirillum*
  3. Filament long, spiral, flexible......................*Spirochaete*
B. Cells united into a glairy family..............................*Myconostoc*

Zopf (1885, p. 50) differentiated *Vibrio* from *Spirillum* because the former was said to produce spores.

Hueppe (1886) further subdivided these forms on a similar basis as follows:

1. Without endospores.................................*Spirochaete*
2. With endospores.
   a. Without change of cell form upon spore production. *Spirillum*
   b. With change of form upon spore production........*Vibrio*

Winogradsky (1888, p. 105) removed the spirals containing sulphur to a new genus and gave them the name *Thiospirillum*.

Schroeter (1886) differentiates by the following key.

1. Cells stiff (not flexuous). With endogenous spores...*Spirillum*
2. Spirals flexuous.
   a. Cells known only in the form of a long flexuous spiral. Spores not known..........................*Spirochaete*
   b. Cells usually only one half spiral, later growing to spirals. Arthrospores?..............................*Microspira*

Migula (1894 p. 237) separated the genera of the *Spirillaceae* as follows:

I. Cells stiff, not flexuous.
   a. Cells non-motile..................................*Spirosoma*
   b. Cells motile, with flagella.
      1. Cells with 1, rarely 2 or 3 polar flagella......*Microspira*
      2. Cells with tuft of polar flagella..............*Spirillum*

II. Cells flexuous......................................*Spirochaete*

Lehmann and Neumann (1896) subdivide the *Spirillaceae* as follows:

I. Cells short, slightly bent, with one or two polar flagella...*Vibrio*
II. Cells long, spirally bent, rigid, usually with a tuft of polar flagella...*Spirillum*
III. Cells flexible, long, spiral, coiled filaments, flagella unknown. *Spirochaeta*

Blanchard (1906, p. 1) removed the genus *Spirochaeta* to the protozoa, and separated the bacterial genera as follows under the heading *Spirobacteria*.

A. Non motile..*Spirosoma*
B. Motile
1. Cells rigid, forming a segment of a circle or united into spirals, 1, 2 or 3 polar flagella..................................*Vibrio*
2. Cells definitely spiral:
a. With endogenous spores. Flagella not polar. *Spirobacillus*
b. With or without endogenous spores. Flagella polar *Spirillum*

The following generic names have been proposed for spiral organisms.

Spirillum Ehrenberg, 1830, p. 38
Myconostoc Cohn, 1875, p. 183
Pacinia Trevisan, 1885, p. 83
Microspira Schroeter, 1886, p. 168
Pseudospira De Toni and Trevisan, 1889, p. 1018
Euspirillum De Toni and Trevisan, 1889, p. 1009
Euspirosoma Migula, 1900, p. 955
Liquidovibrio Jensen, 1909, p. 333
Solidovibrio Jensen, 1909, p. 333
Paraspirillum Dobell, 1911, p. 94
Vibrio Mueller, 1773, p. 39
Spirodiscus Ehrenberg, 1838
Spiromonas Perty, 1852, p. 171
Spirulina Hueppe, 1886, p. 146
not *Spirulina* Turpin, 1827
not *Spirulina* Cohn, 1853
Spirobacillus Metschnikoff, 1889, p. 61
Streptospirillum Billet, 1890, p. 24
Spirosoma Migula, 1894, p. 237
Sporospirillum Jensen, 1909, p. 340

The generic name *Spirulina* is invalid because of prior use for another genus of plants.

The species of the following genera cannot be identified from the description. *Myconostoc*, *Spirodiscus*, *Spiromonas*, *Streptospirillum*, *Spirobacillus*, *Sporospirillum*.

The following are subgeneric terms: *Euspirillum*, *Euspirosoma*, *Pseudospira*.

The following genera are probably not invalid for any of the preceding reasons.

Liquidovibrio, Microspira, Pacinia, Paraspirillium, Solidovibrio, Spirillum, Spirosoma, Vibrio.

The following key will serve to differentiate the genera which may be recognized at present.

Key to genera of Spirillaceae

A. Cells not larger at center, not tapering.
  1. Cells usually short, only a segment of a spiral. One or rarely two or three polar flagella....................Genus 1. *Vibrio*
  2. Cells longer, usually with a tuft of polar flagella. Genus 2. *Spirillum*

B. Cells enlarged at center and tapering..............Genus 3. *Paraspirillum*

Genus 1. **Vibrio** Mueller, 1786 p. 39 emended

Synonyms

Pacinia Trevisan, 1885, p. 83
Pseudospira De Toni and Trevisan, 1889, p. 1018
Microspira Schroeter, 1886, p. 168
Liquidovibrio Jensen, 1909, p. 333
Solidovibrio Jensen, 1909, p. 333

Short, bent rods, sometimes almost straight. Motile by means of a single (rarely 2 or 3) polar flagellum. Aërobic, and facultative. Grow well on ordinary media. Frequently liquefy gelatin. Not enlarged near center. No spores. Usually gram negative.

The type species is *Vibrio cholerae.* The generic name *Vibrio* has been used in several senses by different writers. Most of the species included in this genus by writers before the time of Cohn are now definitely placed in other genera or cannot be identified. Pacini (1851) used the designation *Vibrioni of cholera,* though it is not certain that he actually saw the organism causing the disease.

The *Vibrio* of Müller was any very simple, "terete, elongate worm." Ehrenberg (1838) defined the genus to include straight flexuous rods. Cohn (1872, p. 178) emended the genus to include organisms characterized by a wavy motion of the fila-

ments, the rotation of which gives the appearance of sinuous motion, this character indicating transition to the spiral bacteria. Zopf (1885, p. 61) definitely designates the genus as spiral. Some authors retained the older idea of the genus, and introduced new generic names as *Pacinia* and *Microspira*, others used the generic name *Vibrio* for short spirals. The latter conception is held by Loeffler, Fischer, Lehmann and E. F. Smith and is the one adopted here.

Vuillemin (1913, p. 518) concludes that *Vibrio* used as a generic name should be suppressed because of its varied meanings. He would use the generic name *Microspira*. However, if *Vibrio* should be abandoned, *Pacinia* Trevisan would definitely have priority.

Genus 2. **Spirillum** Ehrenberg 1830, p. 38 emended, Migula, 1894, p. 237.

Synonym:

Spirosoma Migula, 1894, p. 237

Cells definitely spiral, not enlarged at center, motile by means of 5 to 20 polar flagella, or non-motile. Not readily cultivated in ordinary culture media.

The type species is *Spirillum undula* (Müller) Ehrenberg.

Genus 3. **Paraspirillum** Dobell 1911, p. 99

Cells spiral or S shaped, like spirilla, variable in thickness, with a well marked thickening toward middle of the body, and tapering toward the ends, a much elongated and spirally twisted spindle. Motile by means of flagella.

The type species is *Paraspirillum vejdovskii* Dobell.

Family IV. **Nitrobacteriaceae** Fam. nov.

Cells spherical or rod shaped, motile or non-motile, not growing on ordinary laboratory media in the presence of organic matter. Securing growth energy primarily by the oxidation of ammonia to nitrites or of nitrites to nitrates.

The genera may be differentiated by the following key:

Key to the genera of Nitrobacteriaceae

A. Cells rod shaped.
  1. Oxidizing ammonia to nitrous acid. Motile. Genus 1. *Nitrosomonas*
  2. Oxidizing nitrous acid to nitric acid.....Genus 2. *Nitrobacter*

B. Cells spherical...Genus 3. *Nitrosococcus*

Genus 1. **Nitrosomonas** Winogradsky, 1892, p. 127

Synonym:

Nitromonas Winogradsky

Cells rod shaped. Motile. Not growing readily on organic media. Oxidizing ammonia to nitrates.

The type species is *Nitrosomonas europoea* Winogradsky.

Genus 2. **Nitrobacter** Winogradsky, 1892, p. 127

Cells rod shaped, non-motile, not growing readily on organic media, oxidizing nitrates to nitrates.

Winogradsky named no species, although one was described. It may be termed *Nitrobacter winogradski* and regarded as the type species.

Genus 3. **Nitrosococcus** Winogradsky, 1892, p. 127

Cells spherical. Not growing readily on organic laboratory media. Oxidizes ammonia to nitrites.

Winogradsky termed the single species "les microbes nitreux de nouveau monde" but without a species name. It may be termed *Nitrosococcus americanus* and regarded as the type.

REFERENCES

BLANCHARD, R. 1906 Spirilles, spirochetes et autres microörganisms a corps spirale. La Semaine Medical, **26**, 1.

BILLET, A. 1890 Contribution a l'etude de la morphologie et du developpement des Bacteriacees. Extrait du Bulletin scientifique de la France et de la Belgique. Paris.

COHN, FERDINAND 1872 Untersuchungen über Bakterien. Beitrage zur Biologie der Pflanzen **1** (Heft 1), 127–244.

COHN, FERDINAND 1875 Untersuchungen über Bakterien II. Beitrage z. Biologie d. Pflanzen **1** (Heft 3): 141–208.

Ehrenberg, C. G. 1830 Beitrage zur Kenntniss der Organisation der Infusorien und ihrer geographischen Verbreitung. Abhandlungen d. Berl. Akad. pp. 1–88.

Ehrenberg, C. G. 1838 Die Infusionsthierchen als vollkommende Organismen. Leipzig.

De Toni, J. B. and Trevisan, V. 1889 Schizomycetaceae Naeg. in Saccardo, P. A. Sylloge Fungorum **8**, 923–1087.

Dobell, C. Clifford 1911 Paraspirillum vejdovskii n. g. n. sp. a new bacterial form. Arch. f. Protistentk. **24**, 97.

Hueppe, F. 1886 Die Formen der Bakterien und ihre Bezeichnungen zu den Gattungen und Arten. Wiesbaden.

Jensen, Orla 1909a Die Hauptlinien des natürlichen Bakterien systems. Centralbl f. Bakteriol. Abt. 2, **22**, 305–346.

Jensen, Orla 1909b Vorschlag zu einer neuen bakteriologischen Nomenklatur. Centralbl. f. Bakteriol. Abt. 2, **24**, 477.

Lehmann, K. B. and Neumann, R. 1896 Atlas und Grundriss der Bakteriologie. München. Vols. I and II.

Metschnikoff, Elie 1889 Contributions a l'etude du pleomorphisme des bacteriens. Ann. de l'Inst. Pasteur **3**, 61.

Migula, W. 1894 Ueber ein neuer System der Bakterien. Arbeiten ans dem bakt. Institut der Technischen Hochschule zu Karlshuhe. Bd. **1**, pp. 235–238.

Migula, W. 1900 System der Bakterien. Vol. 1, 1897; vol. 2.

Mueller, O. F. 1773 Vermium Terrestrium et fluviatilium.

Pacini, Filippo 1854 Osservazioni microscopiche e Deduzioni pathologiche sul Cholera Asiatico. Gazz. medica italiana, Toscana. Series 2. **6**, 397–401 and 405–412.

Perty, Maximilian 1852 Zur Kenntniss kleinster Lebensformen, Bern.

Rabenhorst, L. 1865 Flora Europaea Algarum. 1864–1865.

Schröter, J. 1886 Die Pilze. In Cohn, Kryptogamen-Flora von Schlesien.

Trevisan, Vittore. 1879 Prime linee d'introduzione allo studio dei Batterj. italiani. Rendiconti. Reale Istituto Lombardo di Scienze e Lettere IV. Series II. **12**, 133–151.

Trevisan, Vittore. 1885 Caratteri di alcuni nuovi generi di Batteriacee. Atti della Accademia Fisio-Medico-Statistica in Milano. Series 4. **3**, 92–107.

Turpin, P. J. F. 1827 Dict. d'hist. nat. de. Leviault.

Vuillemin, Paul 1913 Genera Schizomycetum. Annales Mycologici. **11**, 526.

Winogradsky, S. 1888 Beiträge Morphologie und Physiologie der Bakterien. Heft. I. Zur Morphologie und Physiologie der Schwefelbacterien. Leipzig.

Winogradsky, S. 1892 Contributions a la morphologie des organismes de la nitrification. Archives de sciences biologiques St. Petersbourg, p. 87.

Zopf, W. 1885 Die Spaltpilze, 3 ed.

THE DIRECT OR BREED METHOD FOR COUNTING BACTERIA IN TOMATO CATSUP, PULP OR PASTE

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The method used for counting bacteria in tomato catsups, pulp or paste depends on the use of the Zeiss blood counter. In this method a drop of catsup diluted with two portions of distilled water is placed in the counting chamber so as to cover the space within the moat. This stands for fifteen minutes before counting and the bacteria in 25 small squares are then counted and an average is obtained for 5 of these small squares. The result is then multiplied by 2,400,000 to find the number of bacteria per cubic centimeter. This method is described by B. J. Howard (1911) in detail.

In counting the number of bacteria per cubic centimeter in a number of samples of catsup I found that this method was not entirely satisfactory, as the bacilli are hard to differentiate from micrococci or various small particles found in the catsup. Micrococci are not counted according to this method as they are so liable to be confused with other bodies such as particles of clay.

It was thought that these sources of error might be overcome by the use of the direct method of counting bacteria devised by Prescott and Breed (1911), and the same technique was used as that described under the "Microscopic Method of Analysis" in Standard Methods of Bacteriological Analysis of Milk. (A. P. H. A., 1916). The catsup is diluted with two parts of sterile water, since this dilution has proven satisfactory in counting most specimens when the Zeiss counting chamber is used. With a sterile pipette calibrated to deliver 0.01 cc. the diluted catsup is deposited on a second glass slide and evenly spread over an area of 1 sq. cm. by means of a sterile needle. After drying, the

ZEISS COUNT			DIRECT COUNT		
Sample	Average per square	Bacilli per cubic centimeter		Average field	Bacilli per cubic centimeter
I...........	13	31,200,000	Tomato catsup	188	56,400,000
	19	45,600,000		172	51,600,000
II...........	12	28,800,000	Tomato catsup	392	117,600,000
	10	24,000,000		312	93,600,000
III...........	30	72,000,000	Tomato catsup	388	116,400,000
	26	62,400,000		324	97,200,000
IV...........	18	43,200,000	Tomato pulp	480	144,000,000
	22	52,800,000		482	144,400,000
V...........	16	38,400,000	Tomato pulp	254	76,400,000
	12	28,800,000		200	60,000,000
VI...........	30	72,000,000	Tomato pulp	840	252,000,000
	26	62,400,000		844	253,200,000
VII...........	21	12,600,000	Tomato catsup	33	9,900,000
	20	48,000,000		45	13,500,000
VIII...........	16	38,400,000	Tomato pulp	56	16,800,000
	24	57,600,000		60	18,000,000
IX...........	19	45,600,000	Tomato catsup	92	27,600,000
	16	38,400,000		92	27,600,000
X...........	28	67,200,000	Tomato pulp	304	91,200,000
	18	43,200,000		352	106,000,000
XI...........	30	72,000,000	Tomato pulp	308	92,400,000
	18	43,200,000		328	98,400,000
XII...........	50	120,000,000	Tomato catsup	272	81,600,000
	40	96,000,000		192	57,600,000
XIII...........	18	43,200,000	Tomato catsup	384	115,200,000
	20	48,000,000		478	143,600,000
XIV...........	30	72,000,000	Tomato catsup	124	37,200,000
	36	86,400,000		84	25,200,000
XV...........	46	110,400,000	Tomato catsup	852	255,600,000
	42	100,800,000		796	238,800,000

slide is immersed in 95 per cent alcohol for one minute to fix the smear, dried in the air, stained with Loeffler's methylene blue for two minutes, washed off in water, dried and examined with the $\frac{1}{12}$ inch oil immersion lens. The microscopic field is then standardized by means of a stage micrometer, with the selection of proper oculars and the adjusting of the draw tube so as to bring the diameter of the whole microscopic field to 0.205 mm. Thirty fields are counted and the average for one field is then multiplied by 3 on account of the dilution of the catsup, and this result is then multiplied by 300,000. The result represents the number of bacteria per cubic centimeter of catsup or other tomato product.

Fifteen samples of tomato catsup or pulp were counted by means of the Zeiss blood counter and by the modified direct count, two counts being done on each sample, and the results being averaged The able opposite gives the results in detail, and a comparison can be made between the results obtained from the Zeiss blood counter and by means of the direct count.

CONCLUSION

An examination of the above table will show that the direct count gives much larger numbers of bacteria than the Zeiss blood counter.

Thus, the advantage of the direct count is, first, that a bacillus can always be distinguished from micrococci or inert material, while one is often in doubt when using the Zeiss counter. Secondly, that micrococci can be counted since they are easily stained and can be noted when this method is used.

It is just as important to count the number of micrococci as bacilli, since the former probably take just as active a part in the deterioration of tomato products as the latter. Micrococci are difficult to distinguish by means of the Zeiss counting apparatus but are easily recognized when the direct method is used.

REFERENCES

American Public Health Association 1915 Am. J. Pub. Health, **6**, 1315.

Howard, B. J. 1911 Tomato Catsup Under the Microscope, Circular No. 68, U. S. Department of Agriculture, Bureau of Chemistry, February 13.

Prescott, S. C., and Breed, R. S. 1911 Centralbl. f. Bakteriol. II, **30**, 337.

BLUE-PRINTING DIRECTLY FROM AGAR PLATES

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This spring in planning, for teaching purposes, a large number of illustrative series of photographs showing comparative bacterial conditions (boiled and unboiled water, effects of disinfectants, milk kept at different temperatures, etc.) we found photographs as expensive as permanent glycerine-agar plates themselves. With amateurs the results are most uncertain, even when a really good camera can be secured; and with the delays incident to developing and printing the Petri dishes often spoil or change materially before new exposures can be made.

We finally hit upon the expedient of using blue-print paper, printing directly from the Petri dish, and omitting the camera entirely. The Petri-dish was placed flat upon the blue-print paper and then exposed to the direct rays of the sun—a method long used for leaf-prints. Occasionally the more transparent colonies were "touched up" by using a fine-tipped brush to apply thick white paint to the under side of the Petri dish. Unless the agar layer is thin and unless the light rays fall at right angles to the dish, "touched up" colonies appear blurred in the prints.

The blue print paper used by architects is sensitive enough for most purposes, and much cheaper than the kind sold by photographic supply houses. We had the large sheets cut into 5-inch squares, the total cost (including the cutting) totalling but $1.65 for 500 squares.

A large board was used for holding the Petri-dishes. To the board were fastened by screws or matting tacks narrow strips of metal (old jig-saw blades, in this case). When these were lifted or swung into position (2 or 3 to a dish) their free tips pressed

upon the edge of the Petri dish and held it firmly against the paper.

Blue-print paper has been used for photographing photogenic bacteria, but this particular application is perhaps new. Teachers will readily recognize the value of this method in permanently recording most of the results for which Petri-dishes are used.

I had hoped to publish photographs of the prints as prepared. The blueprints, however, on account of their color, do not reproduce well enough to show their real value. "Black and white" architects' paper which could be used equally well is imported and not to be procured at this time.

COMPARISON OF LOEFFLER'S COAGULATED BLOOD SERUM AND BLOOD SERUM WITH THE ADDITION OF STERILE OX GALL

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In 1913 Von Drigalski and Bierast reported on the results obtained by the comparative use of Loeffler's original coagulated blood serum plate medium and blood serum medium with the addition of sterile ox gall, in the isolation of the diphtheria bacilli. Drigalski's medium was prepared according to the original formula with the addition of 0.13 parts ox gall. They concluded from their work that the modified Loeffler medium rendered the isolation of diphtheria bacilli more certain and in many cases easier, due to the increase in the number of diphtheria colonies on the plates.

Büsing in 1914 repeated the work and compiled the results of various other workers in the use of Drigalski's serum medium. The following table shows the results collected by Büsing.

Table of results

WORKERS	POSITIVE ON BOTH MEDIA	POSITIVE ON DRIGALSKI MEDIA ONLY	POSITIVE ON LOEFFLER MEDIA ONLY
Drigalski and Bierast, 1913	36	17	0
"Halle"	81	17	0
Seligman	30	9	3
Voelckel, 1913	35	0	4
Schulz, 1913	8	0	2
Grundmann, 1913	70	0	20
Büsing, 1913	79	2	7

There were 384 positive findings with Drigalski's medium and 375 with Loeffler's medium, showing in 420 examinations

only 9 cases in which the Drigalski medium proved more advantageous.

The original work which was carried on in this laboratory in the early part of 1916 consisted in the inoculation, with swabs received with the routine throat cultures, of Loeffler's coagulated serum medium in slants and the simultaneous inoculation of slants of bile serum medium made according to the original formula of Drigalski and Bierast (1913). In this way 400 comparative examinations with the bile and the Loeffler's media were made. One hundred and six examinations gave positive findings on both media. Positive results were obtained on Loeffler's medium only, in 8 examinations, and on the bile media alone, in 4 examinations. Growth of the diphtheria bacilli was more luxuriant on the bile medium in 32 of the 106 positive examinations, but in 5 cases growth was more vigorous on the Loeffler medium.

This work was subsequently repeated, the bile-serum tubes being inoculated first with the swabs, to exclude error due to a lessening in the number of diphtheria bacilli carried over in serial inoculations. The results obtained in 78 comparative tests conducted in this manner showed essentially the same findings as in the first experiment.

Thus the results of our experiment comparing the value of bile-serum and Loeffler serum tubes are in close agreement with those of previous workers who used the plating method. In some instances, in our work, the inhibiting effect of bile upon the common organisms present in the mouth, especially the staphylococcus, was very marked; but in general the modified Loeffler's coagulated serum medium offers no advantage over the plain Loeffler's medium in the isolation and identification of *Bacillus diphtheriae*.

REFERENCES

Büsing 1914 Ueber den Zusatz von Rindergalle zum Löfflerschen Diphtherienährboden. Deutsche med. Wchnschr. **40**, 486–487.

Drigalski und Bierast 1913 Ein Verfahren zum Nachweis der Diphtheriebazillen und seine praktische Bedeutung. Deutsche med. Wchnschr. **39**, 1237–1239.

GRUNDMANN 1913 Erfahrungen über den Gallenährboden bei der bakteriologischen Diphtheriediagnose. Berl. klin. Wchnschr. **50**, 2287–2288.
SCHULZ 1913 Erfahrungen mit dem Galle-Diphtherie-Nährboden nach v. Drigalski und Bierast. Deutsche med. Wchnschr. **39**, 2194–2195.
VOELCKEL 1913 Ueber das Nachweisverfahren der Diphtheriebazillen nach v. Drigalski und Bierast. München. med. Wchnschr. **60**, 1883–1884.

STUDIES RELATIVE TO THE APPARENT CLOSE RELATIONSHIP BETWEEN BACT. PERTUSSIS AND B. BRONCHISEPTICUS[1]

I. CULTURAL AGGLUTINATION AND ABSORPTION REACTIONS

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Mallory and his associates in 1912 and 1913, while attempting to prove the relationship of *Bact. pertussis* to whooping cough by animal inoculations, found that the problem was much more complicated than anticipated, their interpretations being clouded by the introduction into the question of the *Bacillus bronchisepticus*, as a result of its presence in some of the animals used for experimental purposes.

During the discussion of the paper by Mallory the observation was made by Dr. J. L. Rhea, that the lesions in pertussis in the human being, due to the bacterium of Bordet are similar to the lesions in the dog, which result from an infection with *B. bronchisepticus*, the cause of distemper, and that this fact suggested an interesting relationship between the two organisms. Later, Mallory states,

> Further experimental work is evidently needed in order to clear up the subject. The two organisms closely resemble each other morphologically and in cultures on potato blood agar, but can be distinguished by their difference in motility and their alkali production in litmus milk.

Soon after the appearance of the work of Mallory, the writers started some experimental work with the two organisms in question, to determine, if possible, just how close was this relationship which apparently existed between them.

[1] Presented at Eighteenth Annual Meeting of the Society of American Bacteriologists, New Haven, Conn., December 27–29, 1916.

At first the experiments were undertaken with two strains of *Bact. pertussis* which had been cultured since 1911, one having been furnished by the laboratory of Bordet and the other isolated in our own laboratory, and three strains of *B. bronchisepticus* isolated by one of us (N. S. F.), one from a dog in 1908, one from a monkey in 1912 and one from a human subject in 1913. Later on these strains were augmented by ten strains of *Bact. pertussis*, furnished by Dr. Olga R. Povitzky of the New York Board of Health Laboratory, through the courtesy of Dr. Park, and three strains of *Bact. influenzae*, together with one strain of a *Bact. pertussis*-like organism from pertussis sputum, isolated in our own laboratory.

CULTURAL REACTIONS

When first isolated, the *Bact. pertussis* developes slowly and, as a rule, preferably on special media, as reported by Bordet, Woolstein and others. After several months of repeated transplantings, however, its ability to grow on various media gradually increases until it finally presents a growth almost identical to, and nearly as luxuriant as, that of *B. bronchisepticus*, and by that time can be cultured on ordinary media.

It has been found by the writers that the one great difference between the two organisms lies in their power of locomotion; the *B. bronchisepticus* is motile while the *Bact. pertussis* is non-motile, several months of attempting to develop a strain that would give some evidence of motility resulting in failure.

While the cultural reactions have been found practically identical, even to the alkali production in litmus milk, contrary to the report of Mallory, and the tan color on potato is shared by both, yet, with the *Bact. pertussis*, these reactions are extremely tardy in making their appearance, usually taking about two or three weeks longer than with the *B. bronchisepticus*. At the end of this time, however, it is practically impossible to differentiate between the cultures of the two organisms.

The following outline will show the characteristics of these organisms, in a general way:

	B. BRONCHISEPTICUS	BACT. PERTUSSIS
Morphology	Very small, slender rod, showing bipolar staining	Small, slender rod, showing marked bipolar staining
Gram	Negative	Negative
Motility	Motile	Non-motile
Agar slant	Translucent, filiform growth	Translucent, filiform growth
Bouillon	*Cloudy.* Older growth with heavy, stringy sediment	*Cloudy.* Older growth with heavy, stringy sediment
Potato	*Tan.* Light tan in twenty-four hours to dark tan in three weeks; medium becoming tanned	*Tan.* Light yellow in twenty-four hours to tan in 3–5 weeks; medium tanned.
Litmus milk	*Alkaline.* Slightly blue at the surface in forty-eight hours. This color proceeds downward, becoming very dark greenish blue in about seven days, while the lower part decolorizes	*Alkaline.* In about 6 days the litmus milk begins to decolorize at the dark bottom of the tube, becomes slightly blue in upper portion in four weeks, and in from eight to ten weeks can scarcely be distinguished from *B. bronchisepticus.*
Litmus-lactose agar	Alkaline (forty-eight hours)	Alkaline (four to six days)
Glucose agar	No gas	No gas
Gelatin	Not liquefied	Not liquefied
Indol	Negative	Negative
Nitrites in nitrate broth	Negative	Negative

AGGLUTINATION REACTIONS

The agglutination reactions of these two organisms have presented some very interesting and rather novel phenomena which, to the writers, suggest at least a distant relationship between them.

In the early part of this year Povitzky and Worth reported the results of some agglutination experiments with these organisms, using a *Bact. pertussis* antiserum only, and concluded that,

B. pertussis strains can be specifically identified from hemoglobinophilic bacilli, pertussis-like bacilli and *B. bronchisepticus*. In no instance was there cross agglutination between these organisms—at least not higher than 1 : 40.

Our work has corroborated that of the authors so far as they have gone. Table 1 gives the results of agglutination between anti-

TABLE 1

Agglutination tests between antipertussis serum and heterologous suspensions. Results August 31, 1916. Serum from rabbit 7, treated with Bact. pertussis no. 0363 (Bordet)

DILUTIONS	SUSPENSIONS OF: Bact. pertussis		B. bronchisepticus			Pertussislike bacteria	Bact. influenzae		
	No. 0363 (Bordet)	No. 0590 (Ferry)	No. 36 (Dog)	No. 123 (Monkey)	(Human)	No. 932558	T. b. No. 2	"S"	No. 3
1–10	–	–	–	–	–	+	–	–	+
1–20	–	–	–	–	–	–	–	–	–
1–40	–	+	–	–	–	–	–	–	–
1–80	+	++	–	–	–	–	–	–	–
1–200	+++	+++	–	–	–	–	–	–	–
1–400	+++	+++	–	–	–	–	–	–	–
1–800	+++	+++	–	–	–	–	–	–	–
1–1600	+++	+++	–	–	–	–	–	–	–
1–2000	+++	+++							
1–3200	+++	+++							
1–6400	++	+++							
1–10000	+	++							
1–20000	–	+							
1–40000	–	–							
Control	–	–	–	–	–	–	–	–	–

This table and the following one illustrate the presence of pro- agglutinoids in *Bact. pertussis* and *B. bronchisepticus* antiserum making it necessary to test all normal and immune sera in dilutions higher than 1–80.

pertussis serum and suspensions of *Bact. pertussis*, a pertussis-like bacillus, *B. bronchisepticus*, and three hemoglobinophilic bacilli. But on using a *B. bronchisepticus* antiserum, the results are entirely different, as the *Bact. pertussis* suspensions agglutinate

nearly as well as the homologous suspension, the *B. bronchisepticus.* The results of such a test are given in table 2 where agglutination tests were made with suspensions of fourteen strains of *Bact. pertussis* and an homologous suspension of *B. bronchisepticus* against antibronchisepticus serum. And finally, table 3 is a summary of all the homologous and cross agglutination tests.

The results therefor show that the *B. bronchisepticus* antiserum will agglutinate both the *B. bronchisepticus* and *Bact. pertussis* antigens, while the *Bact. pertussis* antiserum will agglutinate only its homologous antigen, the *Bact. pertussis.* This reaction was characteristic of every strain of *Bact. pertussis* and *B. bronchisepticus* under observation.

Whether this shows a true relationship between the two organisms, and can be called a specific reaction, is a question.

Preparation of the antigens for the production of the antisera. B. bronchisepticus was grown on plain agar, *Bact. pertussis* no. 0363 (Bordet) and 0590 (Ferry) on ascitic agar, and the New York strains of *Bact. pertussis* and the hemoglobinophilic bacilli on whole-blood (rabbit) agar. Twenty-four hour growths were washed off in 0.2 per cent trikresol in physiologic salt solution—1 cc. to a culture of *Bact. influenzae,* 2.5 cc. to a culture of *Bact. pertussis* and 5 cc. to a culture of *B. bronchisepticus.* The suspensions were thoroughly shaken in a mechanical shaker and, after two days, tested for sterility.

Production of antisera. Before being treated, the serum of each rabbit was tested for agglutinins against all of the organisms under discussion. Any animal showing an agglutination higher than 1–20 against *B. bronchisepticus* antigen or 1–40 against any of the other organisms, was not used.

The rabbits were given three intravenous injections of increasing doses from 0.5 cc. to 2 cc. three days apart and were bled on the fourth day after the last dose; 0.2 per cent trikresol was added to the serum to insure sterility.

Preparation of suspensions for agglutination tests. The suspensions were prepared in general, as follows:

Each culture was transplanted daily for from three days to three weeks—depending upon the organism—on media best suited to it, to insure a good vigorous growth. Then twenty-four hour cultures of

TABLE 2

Agglutination tests showing presence of pertussis agglutinins in antibronchisepticus serum. Results November 7, 1916. Serum from rabbit 1, treated with B. bronchisepticus no. 36 (dog)

DILUTIONS	SUSPENSION OF														
	B. bronchisepticus	Bact. pertussis													
	No. 36 (Dog)	No. 0363 (Bordet)	No. 55	No. 93	No. 95	No. 98	No. 100	No. 109	No. 110	No. 114	No. 154	No. 163	No. 248	No. 251	No. 253
1–10	++	++	++	−	+	+	+	+	++	+	+	−	+	−	+
1–20	+++	++	+++	+	++	++	+	++	++	+	+	−	+	−	++
1–40	+++	+++	+++	++	+++	+++	++	++	+++	+	++	+	++	+	++
1–80	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++
1–200	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1–400	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1–800	+++	+++	+++	+++	++	+++	++	+++	++	+++	+++	+++	++	++	+++
1–1600	+++	+	++	+	+	++	+	++	+	+++	++	+	+	+	++
1–2000	+++	−	−	+	−	+	+	+	−	+++	+	+	−	−	+
1–3200	+++	−	−	−	−	−	−	−	−	+	−	−	−	−	−
1–6400	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1–10000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1–20000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1–40000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 3

Agglutination tests of B. bronchisepticus, Bact. pertussis, and Bact. influenzae suspensions against homologous and heterologous sera

ANTI-SERA	RABBIT NO.	SUSPENSIONS: B. bronchisepticus: No. 36 (Dog)	No. 123 (Monkey)	(Human)	Bact. pertussis: No. 0363 (Bordet)	No. 0590 (Ferry)	No. 55	No. 93	No. 95	No. 98	No. 100	No. 109	No. 110	No. 114	No. 154	No. 163	No. 248	No. 251	No. 253	Pertussis-like bac.: No. 032558	Bact. influenzæ: T.b.2	"S"	No. 3
B. bronchisepticus																							
No. 36	1	1-6400	1-6400	1-6400	1-1600	1-800	1-1600	1-2000	1-1600	1-2000	1-2000	1-2000	1-1600	1-3200	1-2000	1-2000	1-1600	1-1600	1-1600	1-10	1-40	—	—
	2	1-10000	1-20000	1-10000	1-400	1-400	1-2000	1-800												1-20	1-10	—	1-20
No. 123	3	1-6400	1-10000	1-20000	1-3200	1-3200	1-20000	1-6400												1-20	1-10	—	—
	4	1-10000	1-10000	1-20000	1-3200	1-6400	1-6400	1-3200	1-2000	1-6400	1-3200	1-6400	1-3200	1-3200	1-6400	1-6400	1-3200	1-3200	1-6400	1-20	—	—	1-80
(Human)	5	1-2000	1-3200	1-6400	1-800	1-2000	1-800	1-800	1-800	1-2000	1-1600	1-2000	1-2000	1-1600	1-2000	1-2000	1-800	1-800	1-2000	—	—	—	1-10
	6	1-10000	1-10000	1-10000	1-800	1-800	1-800	1-800												1-10	—	—	1-10
Bact. pertussis:																							
No. 0363	7	—	—	—	1-10000	1-20000	1-10000	1-20000	1-800	1-10000	1-10000	1-20000	1-10000	1-20000	1-20000	1-20000	1-10000	1-10000	1-20000	1-10	—	—	1-10
	8	1-80	1-80	1-80	1-10000	1-20000	1-40000	1-20000												—	—	—	1-20
No. 0590	9	—	1-10	—	1-6400	1-6400	1-20000	1-10000												1-20	1-10	—	—
	10	1-10	1-10	—	1-6400	1-10000	1-6400	1-10000												1-20	—	—	1-10
No. 55	19	—	—	—		1-40000	1-20000																
No. 93	20	—	—	—		1-20000		1-20000															
No. 95	21	—	1-10	1-10		1-20000			1-3200														
No. 98	22	—	—	—	1-20000					1-20000													
No. 100	23	1-20	1-40	1-20	1-20000						1-20000												
No. 109	24	—	—	—	1-10000							1-10000											
No. 110	25	1-10	1-10	1-10		1-10000							1-20000										
No. 114	26	1-10	—	—		1-20000								1-20000									
No. 154	27	1-10	1-10	—		1-20000									1-20000								
No. 163	28	1-10	1-10	1-20		1-20000										1-20000							
No. 248	29	1-20	1-20	1-20		1-10000											1-20000						
No. 251	30	1-40	1-80	1-40	1-2000													1-3200					
No. 253	31	1-20	1-10	1-10	1-6400														1-20000				
Bact. pertussis-like:																							
No. 032558	11	1-10			—	—														1-20000	—	—	1-10
Bact influenzae:																							
T.b.2	13	—	1-20	—	—	—	—	1-10												1-10	1-3200	1-20	1-80
"S"	15	1-20	1-20	1-10	1-10	1-20		1-20												1-40	1-200	1-6400	1-200
No. 3	17	1-20	1-20	1-20	1-20	1-20		1-40												1-20	1-10	1-20	1-1600

these were planted on plain agar in quart whiskey flasks—except in the case of *Bact. influenzae* for which whole-blood agar was used. It has been found advantageous to use agar in whiskey flasks, as this method not only gives a larger amount of suspension for less labor, but it also gives a far heavier and healthier growth than when tubes are used, probably because of the greater supply of media, moisture and air. An abundant growth of *Bact. pertussis* can be obtained on plain agar in flasks when it will grow only slightly or not at all on plain agar in tubes. And a young, vigorous growth is necessary to the production of homogeneous suspensions. Also in using agar without either ascitic fluid or blood, all possibility of clumping from this source is avoided. In the case of the influenza bacillus it is necessary to use blood agar to obtain any growth. Just enough whole blood is added to agar to insure growth and it is used before any hemolysis takes place, in order that the suspension may contain as little blood as possible.

The flasks were incubated for from eighteen to twenty-four hours and the growth washed off with 0.5 per cent formalin in physiologic salt solution. The suspensions were then shaken for a few hours and later, after being tested for sterility, were filtered through paper and standardized to about 2000 million per cubic centimeter.

With this technique, homogeneous suspensions of all the organisms used, were produced.

Agglutination tests. In carrying out the tests, the serum was diluted with physiologic solution and each tube contained 0.5 cc. suspension plus 0.5 cc. diluted serum. The tests were all macroscopic and were incubated at 37°C. for twenty-four hours. (+++) represents complete agglutination with fluid clear; (++) partial agglutination with marked clumping, but fluid not entirely cleared up; (+) slight agglutination, but still with positive clumping; and (−) no clumping, no clearing.

Specially graduated 1 cc. pipettes were used for making the serum dilutions and a different pipette was used for each dilution. All glassware used in connection with the tests was clean and sterile.

AGGLUTINATION REACTIONS WITH SERUM FROM DISTEMPER RABBITS AND DOGS

In testing apparently normal rabbits for agglutinins, before beginning inoculation for the production of antisera, we found that if a serum agglutinated *B. bronchisepticus* in a dilution higher

than 1–20, it also agglutinated *Bact. pertussis* and generally in higher dilutions. In one instance when there was no agglutination against *B. bronchisepticus*, the serum agglutinated *Bact. pertussis* in a dilution of 1–400 (rabbit E) (see table 4).

A few of these rabbits subsequently developed symptoms of distemper; the others may have recovered from an attack.

Sera from only two distemper dogs have been tested, but, with these, similar results were obtained. As in rabbits, agglutinins for *Bact. pertussis* were manifest in higher dilutions than for *B. bronchisepticus*. These dogs exhibited typical symptoms of distemper and were in the later stages of the disease.

Dog 1 agglutinated *B. bronchisepticus* no. 36 (dog) at 1–20; and *Bact. pertussis* no. 0363 (Bordet), 1–400 and no. 93, 1–1000. Dog 2 agglutinated *B. bronchisepticus* no. 36 (dog) at 1–80; and *Bact. pertussis* no. 0363, 1–1000 and no. 93, 1–1000.

On absorption with *B. bronchisepticus* the agglutinins for that organism are removed, while the agglutinins for *Bact. pertussis* are affected little or not at all. On absorption with *Bact. pertussis*, the pertussis agglutinins are removed, while those for *B. bronchisepticus* are unaffected.

ABSORPTION REACTIONS

Upon submitting to absorption tests those antiserums which were produced by the injection of *B. bronchisepticus* antigen into rabbits, and which were found to agglutinate both the *B. bronchisepticus* and *Bact. pertussis* suspensions, the following results were obtained.

Upon absorbing with *B. bronchisepticus* suspension, the agglutinins for *B. bronchisepticus* (the major agglutinins) were absorbed, but the *Bact. pertussis* agglutinins (the minor agglutinins) were still intact (table 5), and an absorption with *Bact. pertussis* was necessary before they were neutralized. In other words, the *B. bronchisepticus* antigen stimulated the formation of both *B. bronchisepticus* and *Bact. pertussis* agglutinins, but contrary to what one would expect, was not able to absorb the *Bact. pertussis* agglutinins, (the minor agglutinins). It was also found, in the

TABLE 4

Agglutinin reaction in rabbits not used for production of anti-sera

DILUTIONS	RESULTS																	
	Rabbit A		Rabbit B		Rabbit C		Rabbit D		Rabbit E		Rabbit F		Rabbit G		Rabbit H		Rabbit I	
	B. bronchisepticus no. 36	B. pertussis no. 55	B. bronchisepticus no. 36	B. pertussis no. 55	B. bronchisepticus no. 36	B. pertussis no. 109	B. bronchisepticus no. 36	B. pertussis no. 114	B. bronchisepticus no. 36	B. pertussis no. 163	B. bronchisepticus no. 36	B. pertussis no. 163	B. bronchisepticus no. 36	B. pertussis no. 248	B. bronchisepticus no. 36	B. pertussis no. 251	B. bronchisepticus no. 36	B. pertussis no. 253
1–10	+++	−	+++	+	+++	+	+++	++	−	−	+++	−	+++	++	+++	−	+++	−
1–20	+++	+	+++	++	+++	++	+++	++	−	−	+++	−	++	++	+++	−	+++	+
1–40	+++	+++	++	++	++	++	+++	+++	−	+	++	+	++	++	++	++	++	++
1–80	++	+++	++	+++	+	+++	++	+++	−	++	+	++	+	+++	++	+++	++	+++
1–200	+	+++	+	+++	−	+++	++	+++	−	+++	+	+	+	+++	+	+++	++	+++
1–400	−	+++	−	+++	−	+	−	+++	−	++	−	−	−	+	−	++	−	+++
1–800	−	+++	−	+	−	−	−	+	−	−	−	−	−	−	−	+	−	++
1–1600	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

case of dogs suffering with distemper, that the serum agglutinated *Bact. pertussis* antigen in higher dilutions than the *B. bronchisepticus* antigen. Absorption with *B. bronchisepticus* antigen took out only the *B. bronchisepticus* agglutinin, while it was necessary to absorb with *Bact. pertussis* antigen before the *Bact. pertussis* agglutinin was neutralized (see table 7). The *Bact. pertussis* agglutinins, therefore, were fixed or stable, as far as the *B. bronchisepticus* was concerned, but absorbable for the *Bact. pertussis,* and this type of an agglutinin, which can be produced

TABLE 5

Serum from rabbit 1, treated with B. bronchisepticus absorbed (1–40) with B. bronchisepticus

DILUTIONS	AGGLUTINATION			
	Before absorption		After absorption	
	B. bronchisepticus No. 36	Bact. pertussis No. 0590	B. bronchisepticus No. 36	Bact. pertussis No. 0590
1–80	+++	+++	++	+++
1–200	+++	+++	–	+++
1–400	+++	+++	–	+++
1–800	+++	++	–	++
1–1600	+++	–	–	–
1–2000	+++	–	–	–
1–3200	++	–	–	–
1–6400	+	–	–	–
1–10000	–	–	–	–
Control	–	–	–	–

by one antigen to be taken up or absorbed more readily by another, has been called by the writers, for the lack of a better term, a transitive agglutinin.

The antisera were identical to those used for the agglutination tests.

The heavy suspensions used for absorption were made from twenty hour growths on plain agar in whiskey flasks, suspended in .85 per cent salt solution to which had been added 0.5 per cent formalin. About 10 cc. salt solution was used to a flask for *B. bronchisepticus* and 4 cc. for *Bact. pertussis.* The suspensions were shaken over night in a mechanical shaker and then strained through mull. Each strain had

been transplanted daily for several days before using so that very heavy growths were obtained.

Absorption tests. Equal parts of heavy suspension and serum were mixed; incubated at 37°C.; then centrifugalized and the supernatant

TABLE 6

Absorption tests with B. bronchisepticus and Bact. pertussis antisera

SERUM	ABSORBED WITH	AGGLUTINATION AFTER ABSORPTION	
		B. bronchi-septicus	Bact. pertussis
Antibronchisepticus.			
No. 36 (dog), rabbit 1 . .	*(Original titre)*	*1–6400*	*1–800*
	B. bronchisepticus no. 36	—	—
	Bact. pertussis no. 0590	1–6400	1–80
No. 36 (dog), rabbit 2. . .	*(Original titre)*	*1–10000*	*1–400*
	B. bronchisepticus no. 36	—	—
	Bact. pertussis no. 0590	1–10000	—
No. 123 (monkey), rabbit 3.	*(Original titre)*	*1–10000*	*1–3200*
	B. bronchisepticus no. 123	—	1–2000
	Bact. pertussis no. 0590	1–10000	1–80
No. 123 (monkey), rabbit 4.	*(Original titre)*	*1–20000*	*1–3200*
	B. bronchisepticus no. 123	—	1–80
	Bact. pertussis no. 0590	1–6400	—
Human, rabbit 5.	*(Original titre)*	*1–6400*	*1–800*
	B. bronchisepticus (Human)	—	1–800
	Bact. pertussis no. 0363	1–6400	—
Human, rabbit 6.	*(Original titre)*	*1–10000*	*1–800*
	B. bronchisepticus (human)	—	1–800
	Bact. pertussus no. 0363	1–10000	1–80
Antipertussis			
No. 0590, rabbit 10.	*(Original titre)*	*1–10*	*1–10000*
	Bact. pertussis No. 0590		—
	B. bronchisepticus No. 36		1–6400

fluid tested for agglutinins. The time of incubation, the number of absorptions and the dilution of the serum were varied in a number of ways. For example, we found that when either antipertussis or antibronchisepticus serum was diluted to 1–40, we obtained a more nearly

complete absorption than when diluted 1–10, which may be due to the fact that complete agglutination does not occur in the lower dilutions with either of the organisms, especially with *Bact. pertussis*. And in the absorption tests, it was noted that clumping and clearing was not so complete at 1–10 or 1–20 as at 1–40.

Again, serum from rabbit 5, treated with *B. bronchisepticus* (Human) was absorbed as many as four times, the dilutions being from 1–5 to 1–40 and the tests being shaken in a mechanical shaker before each incubation, with no effect on the agglutinins for *Bact. pertussis*.

It was found that when antibronchisepticus serum was absorbed with *B. bronchisepticus* sufficiently to remove the agglutinins for *B. bronchisepticus* the agglutinins for *Bact. pertussis* were still unaffected. This could be done by an absorption at 1–40, incubated 24 hours. Table 5 gives the results of such an experiment.

TABLE 7

Absorption tests with serum from distemper dogs

SERUM	ABSORBED WITH	AGGLUTINATION AFTER ABSORPTION		
		B. bronchisepticus no. 36 (dog)	Bact. pertussis	
			No. 0363	No. 93
Dog 1	*(Original titre)*	*1–20*	*1–400*	*1–1000*
	B. bronchisepticus no. 36	—	1–200	1–1000
	Bact. pertussis no. 0363	1–20	—	1–20
Dog 2	*(Original titre)*	*1–80*	*1–1000*	*1–1000*
	B. bronchisepticus no. 36	—	1–800	
	Bact. pertussis no. 0363	1–80	—	

But it required repeated absorption with *B. bronchisepticus* before any marked effect was produced in the pertussis agglutinins, and this happened only with the dog strain.

Finally, the following method was used with the six antibronchisepticus sera. The results of these experiments are given in table 6.

Each serum was absorbed three times with its homologous antigen—that is, serum from rabbit treated with *B. bronchisepticus* (dog) was absorbed with the dog strain—and with *pertussis* antigen as follows:

First absorption, serum 1–10 incubated two hours.
Second absorption, serum 1–20 incubated two hours.
Third absorption, serum 1–40 incubated eighteen hours.

SUMMARY AND DISCUSSION

1. After repeated transplantings *Bact. pertussis* has been found to give the same cultural reactions as *B. bronchisepticus*—the tan growth on potato and the alkaline reaction in litmus mik being the most prominent characteristics. The difference in motility and the tardiness in the reactions of *Bact. pertussis* on culture media are differential characteristics.

2. *B. bronchisepticus* antiserum agglutinates not only *B. bronchisepticus* (1–6400 to 1–2,000), but also *Bact. pertussis* (1–400 to 1–6400).

3. *Bact. pertussis* antiserum on the other hand agglutinates only the pertussis bacillus.

4. There was no cross agglutination between *B. bronchisepticus* and a pertussis-like bacillus or three hemoglobinophilic bacilli. Also, there was no agglutination between *Bact. pertussis* and these organisms.

5. *Bact. pertussis* antiserum of high agglutination titre (1–3200 to 1–20000) has been produced, in rabbits, by three intravenous inoculations (three days apart) of sterile, unheated vaccines of fifteen strains of *Bact. pertussis*.

Povitzky and Worth, in the article cited above, conclude that,

> A strongly agglutinating pertussis serum was best obtained in the rabbit by ten to twelve intraperitoneal inoculations of living cultures at seven-day intervals. Agglutinins are also produced by vaccines, but not as abundantly as by living cultures.

After ten to twelve inoculations of live cultures, their rabbits show a titre of from 2400 to 5000 and one rabbit went to 10000.

Over 50 per cent (9 out of 17) of our rabbits show a titre of 20000, and only two were as low as 3200. The one rabbit in the tables of Povitsky and Worth treated with killed vaccine (heated) shows a titre of 1600 after the sixth inoculation and no agglutination after the fourth. They also say,

Two rabbits, more responsive to vaccines, developed, after a few inoculations, a comparatively high agglutination titre—up to 1000.

6. Sera from rabbits treated wtih *Bact. pertussis* grown on blood media developed marked agglutinins for *Bact. pertussis* grown on plain agar.

This is contrary to Bordet's statement that animals inoculated with *Bact. pertussis* grown on Bordet-Gengou medium develop agglutinins for *Bact. pertussis* grown on the same media, not for the organism grown on plain agar; and is in accordance with Povitzky's and Worth's conclusion that,

> From our experience it would seem that the culture medium influences an agglutinable strain in so far as it affects its growth and best development, not in its production of different kinds of agglutinins.

Also, serum from a rabbit treated with *Bact. pertussis* no 0363 (Bordet) grown on *ascitic agar* for the past three years agglutinates all thirteen strains grown on *blood media* (with only one generation, for suspensions, on plain agar); and sera from rabbits treated with thirteen strains grown on blood media agglutinate the ascitic agar strains.

7. When *B. bronchisepticus* antiserum is absorbed with *B. bronchisepticus* sufficiently to remove the agglutinins for *B. bronchisepticus*, the agglutinins for *Bact. pertussis* are still unaffected. These unaffected or fixed agglutinins have been called by the writers, transitive agglutinins.

Upon repeated absorption, agglutinins for *Bact. pertussis* have been removed by the dog strain of *B. bronchisepticus*, slightly reduced by the monkey strain and affected not at all by the human strain.

It may be that, by the absorption tests, grades of differences are brought out between *B. bronchisepticus* from dog, monkey and human which are not shown in the agglutination tests. A similar differentiation is thought to have been brought out by complement fixation tests. (See article in press by Ferry and Klix).

8. When *B. bronchisepticus* antiserum is absorbed with *Bact. pertussis*, agglutinins for *Bact. pertussis* are removed, but agglutinins for *B. bronchisepticus* are unaffected.

9. When *Bact. pertussis* antiserum is absorbed with *Bact. pertussis*, agglutinins for that organism are removed.

10. When *Bact. pertussis* antiserum is absorbed with *B. bronchisepticus*, agglutinins for *Bact. pertussis* are unaffected.

11. The similar morphology, the identical cultural reactions on differential media, the presence of *Bact. pertussis* agglutinins in artificially produced antibronchisepticus serum and in serum from dogs and rabbits suffering or recovered from distemper, all point toward a close relationship between *Bact. pertussis*, the cause of whooping cough and *B. bronchisepticus*, the cause of distemper.

REFERENCES

Bordet, J. and Sleeswyk 1910 Serodiagnostic et variabilité des microbes suivant le milieu de culture. Ann. d. l'Inst. Pasteur, **24**, 476.

Ferr, N. S. and Klix, H. 1917 Studies relative to the apparent close relationship between Bact. pertussis and B. bronchisepticus. II. Complement fixation tests (in press).

Mallory, F. B. and Hornor, A. A. 1912 Pertussis: The histological lesion in the respiratory tract. J. Med. Research **27**, 115.

Mallory, Hornor and Henderson 1913 The relation of the Bordet-Gengou bacillus to the lesions of pertussis. J. Med. Research **27**, 391.

Mallory, F. B. 1913 The pathological lesion of whooping cough. Boston M. & S. J. **169**, 575.

Povitzky, Olga R. and Worth, E. 1016 Agglutination in pertussis. Archives Int. Med. **17**, 279.

THE GROWTH OF BACTERIA IN PROTEIN-FREE ENZYME- AND ACID-DIGESTION PRODUCTS

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The advantages of protein-free synthetic media for biochemical study were appreciated early in the history of bacteriology. With a medium the chemical composition of which is known it should be possible to determine by exact study the chemical nature of the products of bacterial growth. In the complex peptone and infusion media this is impossible.

Many synthetic media have been devised, most of them containing several inorganic salts, and having glucose or glycerol as the source of carbon, and organic ammonium salts, asparagin or glycocoll as the source of nitrogen. Among the most important of the earlier media were those of Cohn, Nägeli, Fränkel, and Uschinsky. The chief difficulty with all of these, however, is that only a limited number of bacteria will grow in them. The more delicate and fastidious of the pathogenic class apparently do not find proper nutriment in such simple media. *B. diphtheriae* either refuses to develop or grows very slowly. Many other organisms show the same indifference.

With the discovery of the value of protein digestion products, and particularly the amino acids, in animal nutrition, these substances assumed much importance in the field of bacterial metabolism. It has been demonstrated that all proteins must first be reduced by the digestive secretions to the amino acid stage before they can be utilized in animal cell metabolism. Based on a similar assumption, protein hydrolysis products and mixtures of amino acids have been in a few instances used as culture media for bacteria.[1]

[1] For a somewhat comprehensive historical review of this subject the reader is referred to the doctorate thesis of one of the authors (Robinson) in the Yale University Library.

Apparently the first protein-free medium which compared favorably with the universally-used peptone medium was the opsine studied by Dalimier and Lancereaux (1913). Their paper is very suggestive, and in a large measure served as the inspiration and point of departure of the present investigation.

Opsine is a biuret-free product resulting from the combined action of trypsin, erepsin and pepsin on certain protein materials, not named by the authors or manufacturers.[2] It contains the amino acids of the hydrolyzed proteins, also nucleic acid and glucosamine, or their decomposition products. Phosphorus would presumably be present in the nuclein and sulphur in the cystine. The opsine gave a high formol titration for monamino acids. Leucine and tyrosine were detected by their crystals, and tryptophane by the bromine water test. The biuret test for protein was always negative. On this basic medium, with the addition of glycerol and agar, Dalimier and Lancereaux, successfully cultivated not only the more common pathogens and non-pathogens, but also such delicate organisms as the pneumococcus, gonococcus, meningococcus, *B. diphtheriae*, *B. tetani* and *B. tuberculosis* (human, bovine and avian types). These were all carried through several transplants, without any apparent loss of viability. When injected into animals, sterile opsine had no injurious effect.

EXPERIMENTAL

In the present investigation numerous cultural tests were made with opsine as the chief and, as a rule, the only source of nitrogen in the medium. Cultural studies were also carried on with protein-free acid-digestion products of proteins, as will be shown later in the paper.

Opsine as a culture medium

The opsine was obtained from Paris in small metal capsules. In its original state it resembles highly concentrated commercial meat extract, although much lighter in color. It dissolves readily

[2] M. Grémy, Rue de la Tour d'auvergne, Paris.

in water, yielding a brown but perfectly clear solution which is easily and completely decolorized by filtration through animal charcoal. The following tests were made with the decolorized solution.

Different modifications of the biuret method for the presence of proteins gave negative results, thus bearing out the contention of Dalimier and Lancereaux that the product is protein-free. The bromine water test for tryptophane was always negative. This observation is opposed to that of the previous authors, who claimed that tryptophane is present. Millon's Reagent gave a very weak reaction. No tyrosin crystals could be identified upon slow evaporation of a strong solution of the opsine, but leucine crystals were very numerous.

In the Sörensen formol titration, 5 cc. of 1 per cent opsine solution required 3.5 cc. of N/20 NaOH to neutralize it after the addition of neutral formalin. In other words, 1 gram of opsine contained 49 mgm. of nitrogen as monamino acid. A 1 per cent solution of opsine has a reaction with phenolphthalein of about +1.

The first experiments were conducted with the opsine media as prepared by Dalimier and Lancereaux. Since opsine is so readily soluble, the different media were prepared with ease. For opsine bouillon, opsine and sodium chloride were added to the water and the mixture heated for a few minutes, or until solution was complete. The reaction was then adjusted, and the medium boiled over a flame for a few minutes and filtered. In the preparation of agar, bouillon was first made as described, and the desired amount of agar added. The agar medium was always tubed without filtering. Witte's powdered agar was employed. All of the media were sterilized for fifteen minutes at 12 pounds of extra steam pressure.

Inoculations were made from twenty-four hour slant agar cultures, and the growths recorded as follows:

−	No growth
±	Very scanty
+	Scanty
+±	Sparse
++	Moderate
++±	Abundant
+++	Luxuriant growth

This system was employed in all of the experiments. In estimating the growth of any particular organism a mental comparison was made with the average growth of that organism on the corresponding extract-peptone medium. This standard growth was rated as +++. These comparisons are, of course, only roughly approximate. Special care was taken, however, to make the inoculations light and as nearly alike as possible. In duplicate experiments the growths agreed surprisingly well. Only a few of the tables are presented in this paper. For a complete record the reader is referred to the original thesis in the Yale Library.

In the following experiments the media had the same composition as those employed by Dalimier and Lancereaux.

Medium I

Opsine 1.0 per cent
NaCl 0.5 per cent
} faintly acid to litmus

Medium II

Opsine 1.0 per cent
NaCl 0.5 per cent
Glycerol 5.0 per cent
} faintly acid to litmus

Medium III

Opsine 1.0 per cent
NaCl 0.5 per cent
} faintly alkaline to litmus

Medium IV

Opsine 1.0 per cent
NaCl 0.5 per cent
Glycerol 5.0 per cent
} faintly alkaline to litmus

Each of the media was employed with and without agar, the amount of agar in the solid media being 1.5 per cent. As test organisms 12 of the common and representative pathogens, and 5 non-pathogens were used. The following is a summary of the results.

The alkaline was the more favorable of the two reactions for *M. cholerae*, *B. anthracis*, *B. abortus*, *Str. pyogenes* and the diphtheria group, and the acid for *B. pyocyaneus*, *Staph. aureus* and *B. typhi*. On the opsine agar the growths were much better than in the opsine bouillon, being in general almost equal to those on the common meat extract peptone agar, and slightly better than those on a medium of Witte's peptone 1 per cent., NaCl 0.5 per cent, agar 1.5 per cent. Glycerol in the alkaline

bouillon slightly increased the growth of almost all of the organisms, but in the acid bouillon it was more often detrimental. This can probably be attributed to the action of acids produced from it by the organisms. In agar the glycerol nearly always appeared to increase the growth slightly. It was also very favorable to intense pigment production.

The addition of Liebig's meat extract, 0.5 per cent, to neutral opsine agar, yielded considerably better results than the opsine neutral agar alone. The difference between the two was more pronounced at forty-eight hours than at twenty-four. The growths on this extract medium were as a rule almost, or quite, as good as on the common laboratory media. Of course, such a medium would not be protein-free, as Liebig's meat extract has been shown to contain traces of proteoses and peptones.

Some of the more fastidious organisms grew on a faintly alkaline medium of fresh beef infusion agar + 1 per cent opsine almost as well as on meat infusion peptone agar. The following table shows the growths of the third transplants on this medium after thirty hours' incubation:

Gonococcus (287)	+±
Pneumococcus (M)	++ large discrete colonies
Pneumococcus (697)	+± small crowded colonies
Meningococcus (M)	++
Meningococcus (200)	++±
B. pertussis	++±
B. diphtheriae (Y. M. S.)	+++
Str. pyogenes	++±
M. melitensis	+

When glucose (1 per cent) was added to opsine agar, neutral to litmus, the growths were usually less abundant than on the plain opsine agar. In the case of *Streptococcus pyogenes*, *B. pyocyaneus*, and *B. prodigiosus*, however, the reverse was true. Like glycerol, glucose greatly increased and intensified color production by the last two organisms. The inhibition on the part of the glucose was presumably due to acid formation.

Nitrates were reduced to nitrites in decolorized opsine bouillon by the five strains of *B. coli* and the one of *B. aerogenes* tried. The sterile controls were negative. As might be expected

from the negative tryptophane tests, no indol could be detected in coli cultures even in 2 per cent decolorized opsine. There was always good growth, and the peptone water controls of the same organisms were strongly indol-positive. Tests were made after three and five days of incubation.

Unfiltered cultures of *B. coli* in 1 per cent opsine gave the biuret and the Hopkins and Cole protein tests after only two days of incubation. It is interesting to note that in some old *B. coli* cultures which had been kept in the laboratory for about two months, the clear supernatant fluid gave a negative biuret test, while the sediment was positive. This would seem to indicate that in cultures of *B. coli* the protein tests were due solely to the bacterial protein of the cells, and that no soluble protein was formed in the medium, as Uschinsky claimed to be the case with cultures of *B. diphtheriae* in his protein-free medium.

In comparisons of the growths in 1 per cent opsine bouillon before and after decolorization with charcoal there were rather striking irregularities, especially with the diphtheria group. Although, in general, growth was a little slower and scantier in the decolorized medium, one or two strains seemed consistently to thrive better in it. *B. diphtheriae* (No. 8) was slow in development in the decolorized medium, and did not form a film as it did in the undecolorized fluid. It was found that the decolorization resulted in only a very slight increase in the acidity of the opsine, (about +0.1), and a very small decrease in the Sörensen formalin titration for monamino acids. Animal charcoal which had been washed free of chlorides was used. On the whole, then, the undecolorized medium was rather more favorable to growth, although when agar was used the difference was very slight. It is probable that the charcoal removed something else from the medium besides the coloring matter, but no definite conclusion as to the nature of this material was reached. It was shown not to include the monamino acids.

In order to determine whether opsine could be improved as a culture medium by the addition of certain inorganic salts and carbo-hydrates the following substances were added to 2 per cent opsine and to fresh beef infusion + 1 per cent opsine.

	per cent
NaCl	0.5
KH_2PO_4	0.5
Sodium citrate	0.2
$MgSO_4$	0.2
Glucose	0.5
Glycerin	6.0

The media contained 1.5 per cent agar. Plain 1 per cent and 2 per cent opsine agar were also used. All of the media were very faintly alkaline to litmus, and inoculations were made from young agar cultures. The growths were recorded after forty-eight hours' incubation.

The growths on 2 per cent opsine alone were on the whole fully as good as those on 2 per cent opsine plus the above added substances. Every organism out of the large number tried, with the exception of one strain of pneumococcus, grew on opsine alone without beef infusion. The medium containing 1 per cent opsine + beef infusion + the above agents was a little less favorable for the gonococcus and pneumococcus (save strain 697) than 2 per cent opsine + the given mixture, but was decidedly more favorable for *B. suisepticus* and the streptococcus of equine influenza. In the case of the other organisms there was very little difference.

Experiments were also performed to compare the morphology and viability of cultures on opsine agar with those on fresh beef infusion peptone agar. These two media were prepared with reactions faintly alkaline to litmus, 1 per cent opsine, and 1 per cent peptone and 1.5 per cent agar being used. The cultures were transplanted four times on opsine agar and twice on the beef infusion peptone agar, the medium on which they were accustomed to grow. Pathogenic bacteria were incubated at 37° and saprophytic organisms at 24°. Transfers were made every two days. In general, there was little difference in the appearance and luxuriance of the growths on the two media, especially in the last transplants. Differences were distinctly apparent in the following cases: On opsine the growth of *Leptothrix* was wrinkled, while on the control medium it was larger in amount and smooth. *B. suisepticus* and *B. diphtheriae* (K.L.)

grew better on the control. On the other hand, the Hofmann bacillus showed a much better growth on opsine. Cultures of *B. prodigiosus* on opsine were of a good red tinge, but in the control tubes were colorless.

As smears from the opsine and the control cultures were made and stained side by side on the same slide, a careful comparison between the two series as to morphology and staining reaction was possible. With many organisms, the stained preparations from the two media were practically identical as to the morphology and uniformity of the bacteria, and the nature and depth of the stain. In others, however, distinct differences could be detected, which were sometimes in favor of the opsine, and again to the advantage of the control medium. Several Gram-positive organisms showed a tendency to be Gram-negative on the opsine.

In order to see if long continued residence in opsine would induce any changes, these cultures were kept at 16° for twenty-four days, then a fifth series of transplants was made to the opsine and control media respectively, and the organisms stained as before. The results on the opsine were somewhat more favorable than before, although there was no very striking or general change. Without going into much detail, it can be stated in a general way that on opsine *B. coli*, *B. pneumoniae*, *M. cholerae*, *B. avisepticus*, *B. glycobacter* (*peptolyticus*), *Streptococcus viridans*, *Proteus vulgaris* and the diphtheria group were more typical and uniform, while the control medium was the more favorable for *Bact. pullorum*, *Staph. aureus*, *B. prodigiosus*, *B. fluorescens* (*liquefaciens*) and *Proteus zenkeri*. On the whole, the pathogens, especially those that are more difficult to cultivate on ordinary media, as the diphtheria group and *Streptococcus viridans*, were more uniform and typical in morphology, and stained better, when grown on opsine than on the control beef infusion peptone medium.

In order to test the comparative viability of the organisms on the two media, the above mentioned growths (fourth transplants) were kept at 16° and subcultures made at different intervals to beef infusion and beef extract peptone agar.

Stains made at the end of twenty-four days showed that all

were pure cultures. Wherever a subculture failed to develop, the operation was repeated, heavier inoculations being made. This often changed the result.

It was found that *B. suisepticus* was alive on both media after sixteen days, but dead after twenty-four days; *B. acne* (51) was alive on both at twenty-four days, and on opsine at forty days, the control being contaminated. *Streptococcus viridans* died on the control before sixteen days, but was still viable on opsine after two months. After twenty-four days, *Leptothrix* was dead on the opsine, but living on the control, while at forty days both cultures were dead. *B. diphtheriae* (strains A, Y.M.S., and 8m), were dead on opsine at two months, forty days and twenty-four days respectively. All were alive on the control medium after two months.

B. hofmannii could not be subcultured from opsine after forty days, and from the control after two months. All the other forms were still alive on both media at the end of two months (16°).

Viability tests were also made on 1 per cent *decolorized* opsine agar, inoculated from the above fourth transplant. No controls were used.

All of the organisms grew, although in a few instances not quite as well as on the undecolorized opsine agar. After one month at 16° all of the cultures save *Leptothrix*, *B. diphtheriae* (strains a, Y.M.S., and 8m.), and *Actinomyces* were still alive. At the end of one and one-half months, the cultures of *B. suisepticus* and *B. hoffmannii* had died.

Growth, morphology and viability of menigoccocus, pneumococcus gonococcus and B. influenzae on opsine agar and on fresh beef infusion peptone agar used as control

The two strains of meningococcus employed grew about as well on opsine (1 per cent) agar as on the control peptone agar, and remained viable equally as long if not longer. Stains from the control cultures showed a great majority of peculiar involution forms of varied types, mostly thread-like filaments or spindle-

shaped forms, and very few typical diplococci. On the other hand, the stains from the opsine cultures contained very few involution forms, and were almost indistinguishable from those on blood serum. All were Gram-negative. One strain (meningococcus m) remained alive on 2 per cent opsine agar for four months at 18°. Both strains always grew on 1 per cent opsine agar in twenty-four hours, but a little less luxuriantly than on the 2 per cent medium.

Five out of the six strains of pneumococcus employed grew on 2 per cent opsine agar, four of them nearly as well as on the control medium. The fifth was carried through four transplants on 1 per cent opsine agar with rather scanty growth, and was always negative on meat extract peptone agar. In Gram stains the opsine and control cultures were both quite typical, but in the former about half of the organisms were Gram-negative, while on the control all were positive.

Viability tests on opsine agar and control meat infusion peptone agar

The meningococcus, *B. influenzae* and *B. pertussis* were carried through 12 transplants, and the pneumococcus, gonococcus and *M. catarrhalis* (a.m.) through 8 transplants on opsine. The growths on the last series were compared with those on the control medium, both being 48 hours old. The meningococcus and *B. pertussis* were grown on 1 per cent, the others on 2 per cent opsine. All of the media were faintly alkaline to litmus.

Growths at forty-eight hours

	OPSINE	CONTROL
Pneumococcus (80)	++	++
Pneumococcus (690)	++±	++
Gonococcus (287)	±	+±
M. catarrhalis (a.m.)	+++	+++
B. influenzae	+±	++
B. pertussis	++±	++±
Meningococcus (m)	++	++
Meningococcus (200)	++±	++±

The meningococcus cultures were kept at 18°, the others in the refrigerator, and subcultures were made on the control medium at various intervals. After seven days all were still alive, but after seventeen days pneumococcus no. 80 had died on both media and pneumococcus no. 690 on the opsine. At twenty-five days, pneumococcus no. 690 was still alive on the control medium, but gonococcus no. 287 had died on both media and *M. catarrhalis* on the opsine. At the end of one month pneumococcus no. 690 and *M. catarrhalis* had died on the control also.

In another lot kept at 16° for twenty-eight days, pneumococcus no. 80 was still alive on both media, pneumococcus no. 690 on opsine, but not on the control, gonococcus no. 287 on the control but not on the opsine, *M. catarrhalis* on neither, and the others on both.

On decolorized opsine the growth and viability of these organisms was distinctly less than on the undecolorized medium.

Acid-digestion products as culture media

Casein, milk albumin and edestin were the proteins chosen for the preparation of the digestion products. Casein contains all of the well-known amino acids with the exception of glycocoll and possibly cystine. In its crude form it is cheap and readily obtainable. Commercial milk albumin also is inexpensive, but it contains a limited number of amino acids, alanine, valine, leucine, aspartic acid, glutamic acid, phenylalanine, tyrosine, proline and tryptophane having been found. Edestin is a representative of the group of vegetable proteins, and contains all of the amino acids save perhaps oxyproline.

The above proteins were hydrolyzed with hydrochloric acid and the products prepared for us by Professor Lafayette B. Mendel, as follows.

Casein product A. One hundred grams of casein were heated with 400 cc. of concentrated HCl for a few hours on the water bath, under a reflux condenser. There was much charring, so the mixture was diluted with 150 cc. of water and heated over a free flame for eight hours. A large amount of humus residue was filtered off. The filtrate

was biuret-free, and gave a strong ninhydrin test for amino acids. It was subjected to prolonged evaporation on a water bath, with frequent renewal of water.

Casein product B. 40 grams of crude casein were boiled for eight hours with 200 cc. of 9 per cent HCl under a reflux condenser over a free flame. There was practically no dark residue. The liquid was biuret-free, and gave a strong ninhydrin test. It was heated in an open dish on the water bath until acid vapor ceased to come off.

Casein product C. 50 grams of the same casein used in B were boiled for eight hours with 200 cc. of 10 per cent HCl over a free flame, with a reflux condenser. The final solution was subjected to distillation, with frequent addition of water. An abundance of fatty-acid-like material came off in the distillate. This product (the distillation liquid residue) was a-biuretic, but gave a strong reaction for amino acids.

Edestin product. 40 grams of pure re-crystallized edestin were heated over a free flame with 10 per cent HCl (reflux condenser) until a biuret test was no longer given. The solution was then heated for several hours in an open dish, to remove as much hydrochloric acid as possible.

Lactalbumin product. 100 grams of crude commercial milk albumin containing about equal amounts of calcium phosphate and protein were heated two to three hours with 200 cc. of 10 per cent HCl. This treatment brought about a solution of most of the calcium phosphate. The residue of protein was filtered off and heated with 200 cc. of 10 per cent HCl until no biuret test could be obtained. The resulting solution was then subjected to prolonged heating in an open vessel, to remove as much as possible of the HCl. Finally the liquid was neutralized with NaOH for the precipitation of any remaining calcium phosphate, and filtered. Part of the filtrate was used as such; the remainder was evaporated to a sticky paste.

These products were all of a very dark brown color, but, like the opsine solution, could be easily decolorized with animal charcoal. For the sake of brevity they will hereafter be designated as "Casein A," "Casein B," "Casein C," "Edestin product" and "Lactalbumin product." The biuret test was negative in all, even by the most delicate methods. As might be expected from the severity of the treatment, all tests for tryptophane were also negative. When, however, a small amount of a weak tryptophane or peptone solution was added, a positive result was obtained, showing that the negative tests were not due to inter-

ference. Tyrosine and leucine crystals were detected in all of the solutions, on evaporation, and glutamic acid crystals in the "Lactalbumin product." Millon's test was always positive.

It was found, by evaporating to as near dryness as possible, that a medium containing 5 per cent of any of these solutions consisted of about 1 per cent solid matter. Sörensen's formalin titration for monamino acids was tried on the casein products. 5 cc. of a 5 per cent solution of "Casein A" required 4.1 cc. of $\frac{N}{20}$ NaOH to neutralize it after the addition of the neutral formalin. With "Casein B" and "Casein C" the figures were 2.9 cc. and 3.7 cc. of $\frac{N}{20}$ NaOH respectively. In an ammonia determination by Folin's method, 10 cc. of the original "Casein C" solution was found to contain 25 mgm. of N as NH_3. The other solutions were not tested.

All of these products could be decolorized and made up into culture media in the same easy and rapid way as opsine. Considerable NaOH was necessary to neutralize the media. It was found that 100 cc. of a 5 per cent "Casein C" solution required about 0.8 cc. of $\frac{N}{1}$ NaOH to make it neutral to litmus, so there was approximately 0.5 per cent of NaCl formed in the process. The other media did not differ very materially from this.

Bacterial Growth in Casein Products

In a medium of "Casein A" only, decolorized and of 10 per cent strength, 17 saprophytes and 14 pathogens, including *B. anthracis*, *B. typhi*, *M. cholerae*, *Bact. pullorum*, *B. diphtheriae*, *B. pseudo-diphtheriae* and *Streptococcus*, were grown; also two streptothrices and two moulds. When the reaction was neutral to phenolphthalein the diphtheria group grew sparsely, while with a reaction of +1 there was no growth. *B. anthracis* and *M. cholerae* also grew better on the neutral media, while *Streptococcus* showed the reverse tendency. Almost all of the other organisms grew quite well on both media. Color production was weak or entirely lacking with *B. prodigiosus*, *Staph. aureus*, *B. ruber* (*Balticus*) and *S. aurantiaca*, but good with *B. pyocyaneus* and *B. fluorescens* (*non-liquefaciens*).

In a medium of "Casein B" (5 per cent), decolorized and neutral to litmus, the same fourteen pathogens as in the last experiment were inoculated. After forty-eight hours there was no growth of *B. diphtheriae* (two strains) and of *Streptococcus*, rather scanty growth of *B. abortus* (Bang) and *B. pseudo-diphtheriae*, and good growth with all of the others. Not so many saprophytes as before were used, but all of those that were tried showed abundant growth.

For the purpose of driving off more of the HCl, some of the "Casein B" solution was evaporated over a water bath to a thick paste, and as nearly as possible to dryness. Agar was prepared from this paste in the same way as opsine agar, using 1.5 per cent of the paste. On the decolorized agar which was faintly alkaline to litmus *B. typhi* (I), *B. paratyphi A* and *B*, *Bact. pullorum*, *Spir. finkler-prior*, *Staph. albus*, *B. diphtheriae* (Y.M.S.), *B. acne* (51), *B. pertussis* and a pathogenic yeast found in tubercular sputum grew almost as well as on peptone meat extract agar. *Streptococcus* (310), *B. diphtheriae* (D), *Glycobacter peptolyticus* and *Proteus zenkeri* showed only a meagre development, and *B. diphtheriae* (K.L.) no growth at all.

These cultures were then kept at 16°C. and subcultures made at different intervals to determine their viability. After five weeks *B. diphtheriae* (Y.M.S. and K.L.), *B. acne* (51), and *B. pertussis* were found to have died. At eleven weeks all of the others were still alive.

Effect of decolorization with animal charcoal

In an experiment to determine whether the decolorizing process had any effect on the nutrient properties, "Casein B" agar was prepared as in the last experiment, one-half of the lot of medium being decolorized. Both the decolorized and undecolorized agar were inoculated at the same time from young cultures.

It was found that in general decolorization of the medium detracts little from its nutrient properties. However, *B. typhi* (10) and *Streptococcus* (urine), which grew scantily on the natural medium, failed to grow at all when the latter was decolorized, and

B. diphtheriae (a) and *B. pseudo-diphtheriae* grew a little less readily after decolorization. The undecolorized cultures were kept fifteen days longer at 16°, and then transferred again to the same medium. All but *B. typhi* (10), *M. catarrhalis* and *B. diphtheriae* (a and 8m) were still alive. The list included the meningococcus, *B. avisepticus*, *B. abortus*, *M. melitensis* and *M. cholerae* (*asiaticae*).

Effect of adding various substances to the digestion products

When 0.5 per cent Liebig's meat extract was added to "Casein B" media, the growths were quite noticeably increased. Pigment production by *Staph. aureus* and *S. aurantiaca* was also improved. On agar containing 0.5 per cent meat extract only, growths were scanty and in the case of *B. diphtheriae*, entirely lacking.

Since Vedder has claimed that starch is a valuable constituent in culture media, this was also added to "Casein B" agar to the amount of 1 per cent. No difference in the growth of the organisms could be noticed, however.

Comparisons were made between the following media, all undecolorized and containing 1.5 per cent agar; (1) "Casein C" 3 per cent, (2) "Casein C" 5 per cent, (3) "Casein C" 7 per cent and (4) "Casein C" 2.5 per cent + edestin product 2.5 per cent + lactalbumin product 2.5 per cent. On media 1 and 2 the growths were not quite so good as on medium 3. The mixture of products proved slightly superior to the other media, *Streptococcus* and *B. diphtheriae* (A) growing scantily on this, and not at all on the others. However, the mixture was not so distinctly superior as had been hoped.

Some of the more difficult organisms to cultivate were also tried out on the following media:

I. "Casein C" 8 per cent.

II. Lactalbumin product paste 2 per cent.

III. "Casein C" 2.5 per cent + lactalbumin product 2.5 per cent + edestin product 2.5 per cent.

All contained 1.5 per cent agar, were not decolorized, and were

made faintly alkaline to litmus. The results are given in the following table:

	FORTY-EIGHT HOURS		
	"Casein C" 8 per cent	Lactalbumin products 2 per cent	Medium III
Gonococcus (287)	±	−	−
Pneumococcus (M)	++	−	−
Pneumococcus (697)	−	−	−
Meningococcus (M)	+±	+±	+±
Meningococcus (200)	++	±	+±
B. pertussis	+++	++	+++
B. diphtheriae (Y. M. S.)	++	+±	+++
M. melitensis	+	−	+++
Streptococcus (urine)	±	±	++±

As was to be expected, medium II gave the poorest results. The gonococcus, pneumococcus, meningococcus and *B. pertussis* grew on at least one of these media (I), with the exception of pneumococcus 697. The same results were obtained with transplants made from these cultures to the same media.

Since tryptophane was found to be absent from all of the acid-digestion products of the proteins used, some experiments were conducted in which tryptophane was added to the different digestion products. The tryptophane exerted very little, if indeed any, influence on the nature and luxuriance of the different growths.

Comparison of growths on Witte's peptone, opsine, lactalbumin product paste, Casein C, and Casein C, edestin and lactalbumin product paste (combined)

Medium I. Witte's peptone 1 per cent, NaCl 0.5 per cent.
Medium II. Opsine 2 per cent.
Medium III. Lactalbumin product paste 2 per cent.
Medium IV. Casein C product 8 per cent.
Medium V. Casein product C 2.5 per cent + edestin product 2.5 per cent + lactalbumin product 2.5 per cent.

All of these media contained 1.5 per cent agar, and were faintly alkaline to litmus. They were all inoculated at the same time and from the same culture tube. The results are shown in the following table.

	I WITTE'S PEPTONE 1 PER CENT	II OPSINE 2 PER CENT	III LACTALBUMIN PRODUCT PASTE 2 PER CENT	IV "CASEin c" 8 PER CENT	V MIXED MEDIUM
B. diphtheriae (A)	+	+++	±	±	+
B. diphtheriae (Y. M. S.)	+	+++	++	++	++±
B. diphtheriae (no. 8)	+±	++	+	++	+±
B. diphtheriae (no. 8m)	+	+++	−	−	+
B. pseudo diphtheriae	++	++±	+±	++±	++
B. abortus (B)	+	++±	+±	++	++
B. pertussis		++±			+±
M. melitensis	±	++±	+±	++	++
M. catarrhalis	+	+++	+	+±	±
Gonococcus (287)	−	+	+	+	±
Pneumococcus (m)	+	+	−	±	−
Pneumococcus (697)	+±	−	−	−	−
Meningococcus (M)	+	+	+	+	+
Meningococcus (200)	−	++	+	+	+
Streptococcus (urine)	±	+++	+	±	++
Streptococcus pyogenes (310)	+	++±		±	+
Streptococcus pyogenes (347)	+±	+++	+±	+±	++±
Streptococcus (I)	±	+		±	+
Streptococcus (IV)	±	++		±	+
Streptococcus (D)		+±		±	+
Starch bacillus (3)		+++		+±	−
Starch bacillus (6)	++±	+++		+±	+
B. acidophilus		++			−
Staphylococcus aureus (II)		+++			±
Staphylococcus aureus (III)		+++			+
Streptococcus pyogenes (VI)		++			

The two strains of a so-called "starch bacillus" were Gram—, spore-forming, starch-digesting organisms, obtained from the intestines of rats fed on a diet of cornstarch. *Staphylococcus* (II and III) and *Streptococcus* (strains I, IV, D and VI), had been freshly isolated from the milk of cows suffering from mammitis.

It is at once evident from the table that the growths on 2 per

cent opsine were strikingly more abundant than those on Witte's peptone (1 per cent). *Gonococcus* no. 287, and *Meningococcus* no. 200 grew on the opsine and not on the peptone, while the reverse was true with *Pneumococcus* no. 697. "Casein C" 8 per cent and medium V, while not as good as the 2 per cent opsine, were still superior to the 1 per cent Witte's peptone. Some organisms grew better on one of these two and some on the other. On the whole, the balance was slightly in favor of medium V. Lactalbumin product paste seemed to have, in a general way, about the same value as the peptone. In two successive transplants to the same media the cultures on "Casein C" 8 per cent and on medium V showed a decided tendency to die out. In the case of "Casein C" 8 per cent there was no growth of the streptococci nor of *Meningococcus* no. 200. On medium V the results were a little better, only two out of the five strains of streptococci dying out, in addition to *Meningococcus* 200. On the other hand, all of the cultures on the opsine medium were thriving well after five transplants on this medium.

GROWTHS IN LACTALBUMIN AND EDESTIN PRODUCTS

A few preliminary experiments demonstrated that the edestin and lactalbumin products were inferior to the casein products as culture media, so little work was done with these two. On the 5 per cent decolorized edestin product in 1.5 per cent agar, neutral to litmus, 7 pathogens and 14 saprophytes were cultivated. *Streptococcus*, *B. abortus* and the diphtheria group failed to grow, while the others did not thrive as well as on the casein products. Pigment production by *B. prodigiosus* and *B. ruber* (*balticus*) was especially striking and intense, and was also good with *Staph. aureus*, *M. cereus* (*flavus*) and *S. aurantiaca*. In the case of *B. pyocyaneus* the color was a deep blue, while cultures of *B. fluorescens* were colorless, which shows that in this medium no fluorescent pigment is produced, only the bluish pyocyanin. When transplanted to the same medium again, *B. ruber* lost its color, while after two or three transfers no change in the others was apparent. As with the other products, the addition of

Liebig's meat extract (0.5 per cent) considerably improved the medium. The color of *B. prodigiosus* cultures was not nearly as brilliant as on the edestin product alone, but the fluorescent pigment was formed by *B. fluorescens* and *B. pyocyaneus*.

It was found that the growths on the lactalbumin product paste were *much* better on 2 per cent of the paste than on 4 per cent, and *slightly* better than on 1 per cent. Too large an amount of this strongly acid paste on neutralization probably formed enough NaCl to be inhibitory. In general, this product was a better culture medium than the edestin, but inferior to the casein product.

THE GROWTH OF ANAEROBES IN THE PROTEIN-FREE DIGESTION PRODUCTS

It was surprising to find that not only *B. tetani*, *B. aerogenes* (capsulatus) and *B. botulinus*, but also the putrefactive organisms, *B. edematis* (*maligni*), *B. anthracis-symptomatici* and *B. putrificus*, grew well on the different protein-free digestion products, especially opsine.

In 2 per cent opsine *B. edematis* (*maligni*) and *B. anthracis-symptomatici* gave very heavy growths in from twenty-four to thirty-six hours. After three days of incubation spores became abundant and only a few bacilli remained. Repeated transplantation in this medium did not apparently affect the viability of these organisms.

In 2 per cent opsine plus 1 per cent glucose the growths were even more luxuriant, but the morphology of the bacilli was not the same as it was in the plain opsine. The bacilli were longer and thinner. Many were granular and stained poorly. Spore formation was indefinitely delayed, and the organisms underwent marked granulation and degeneration.

B. aerogenes (*capsulatus*) grew fairly well in 2 per cent opsine during three days' incubation. No spores were formed in plain or glucose-containing opsine. *B. putrificus* yielded a good growth in plain and in glucose opsine in five days. *B. botulinus* underwent rapid development in two to three days. Spore formation was

quite abundant. Two strains of *B. tetani* grew well in 2 per cent opsine when in association with *Staphylococcus aureus*. Spore production was quite apparent.

For comparison, plain standard bouillon and 2 per cent opsine having the same reaction were inoculated with the same organisms and incubated side by side for five days at 37°C. The results are indicated in the following table.

Growths in opsine and in bouillon; five days' incubation at 37°C.

	OPSINE 2 PER CENT	BOUILLON
B. edematis (*maligni*)	++	+++
B. anthracis-symptomatici (*B. chauvei*)	++	+++
B botulinus	+++	++
B. putrificus (A) + *Staphylococcus*	++	++

It was noticed that spore formation was always more advanced in the opsine cultures than in the bouillon. *B. putrificus* (A) was also grown with *Staphylococcus aureus* aerobically in these two media for the same length of time (five days). On the whole, when in symbiosis with *Staphylococcus aureus* it grew as well in free contact with the atmosphere as in Buchner tubes, and the morphology was about the same in both cases.

All of these anaerobes, except *B. botulinus*, were also tried in 1 per cent opsine + 1 per cent glucose, being grown in Buchner tubes for five days. All grew well. *B. edematis* and *B. anthracis-symptomatici* formed no spores, but quite typical and generally well-stained rods were numerous.

B. bulgaricus and *B. acidophilus* produced a good growth within twenty-four hours in 1 per cent opsine + 1 per cent glucose. No loss of viability was apparent after six transplantations in this medium.

SUMMARY

The protein-free enzyme-digestion product opsine serves as an excellent culture medium for both pathogenic and non-pathogenic bacteria. Practically all of the many organisms employed

grew as well or better in opsine than in Witte's peptone. The cultural characters on the two media were in general the same.

With the exception of the diphtheria group, the different organisms remained viable as long on opsine as on the standard peptone-meat-infusion medium.

A reaction slightly alkaline to litmus was found to be the best for general use. Glycerol increased the growths in alkaline but not in acid media, while glucose seemed as a rule to be somewhat detrimental. Prepared meat extract, and better still, fresh beef infusion, were distinctly favorable. Decolorization with animal charcoal slightly lessened the value of the opsine as a culture medium.

The decomposition products of casein, lactalbumin and edestin obtained by acid hydrolysis were also employed as culture media. Of these the hydrolyzed casein gave the best results, but it was inferior to the opsine. The edestin product was the least satisfactory. It is highly probable that in the hydrolysis of these proteins with hydrochloric acid the treatment is sufficient to destroy many of the intermediate decomposition products which serve so admirably as food for microorganisms.

Bacteria do not require proteins, even in minute quantities, to carry on their normal cultural development, but obtain their sustenance from less complex substances, as for example the amino acids and perhaps some of the simpler polypeptids.

In conclusion we desire to express our sincere gratitude to Prof. Lafayette B. Mendel for the preparation of the different acid-digestion products employed in this investigation, and for his hearty coöperation throughout the entire course of this work.

REFERENCES

Dalimier, R. et Lancereaux, E. 1913 Le milieu de culture d'acides aminés complets pour les microorganismes. Presse Med., 21, 419: Les produits de la proteolyse naturelle et totale comme milieu de culture pour les microorganismes, Arch. de méd. exper. et d'anat. path., **25**, 449–68.

THE CHARACTERISTICS OF BACTERIA OF THE COLON TYPE OCCURRING IN HUMAN FECES

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INTRODUCTION

The great value to sanitary science of a knowledge of intestinal bacteria has stimulated investigation on the colon group until an extensive literature on this subject has accumulated. The striking character of the group is its activity in fermenting carbohydrates and on this most of the attempts at classification have been based. Unfortunately, inexact methods of demonstrating and measuring complicated physiological processes have detracted from the value of some of this work. The fermentation of carbohydrates by microörganisms is evidently a much more complicated process than was formerly supposed. The intermediate and end products are subject to no little variation under the influence of the conditions of the fermentation. Conclusions based on inexact methods are necessarily open to question. This is particularly well illustrated by the history of the Smith fermentation tube, the limitations of which were better recognized by its originator than by many who have followed him in its use. At first great dependence was placed on the volume of gas evolved and the relative percentage of hydrogen and carbon dioxide produced, as determined without taking into consideration the dissolved gas. The results obtained by these methods were so erratic that the fermentation tube has come to be used merely for the determination of fermentation and the variations in the volume and ratio of gases

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have been ascribed to the uncertainties of the fermentation itself rather than to the crudeness of the method. The work of Keyes (1909) and of Keyes and Gillespie (1912) suggested the possibilities in the application of exact methods to the measurement of bacterial fermentations and the value of results so obtained in taxonomic problems.

Our own work has included a study of a collection of colon organisms from milk, one from bovine feces and one from grains. In these investigations we have endeavored to use methods which are as exact as the large number of determinations necessary for statistical treatment will reasonably permit. We first applied exact gas analysis. This has now been supplemented by more rational methods of studying acid fermentation and it is hoped that in time improvements in other cultural tests will become available.

In this paper we give the results of a study of a collection of lactose fermenting bacteria from human feces. This was undertaken primarily to establish the characters of colon cultures from the human intestine.

The failure in the past to secure satisfactory classifications on the basis of fermentation tests has been largely because the characters selected for primary divisions were not of a fundamental nature.

The gaseous fermentation appears to be a basic function of the organisms in question. Even if it is not, it furnishes a basis for primary classification which has established a considerable degree of order, not only among the results of other cultural tests but in the correlation between these tests and the sources of the cultures. We have, therefore, made exact gas determinations on all of the cultures isolated. In addition, we have determined the fermentation of various sugars and alcohols, the formation of indol from tryptophane, the formation of pigment, the liquefaction of gelatin, the carbinol or Voges-Proskauer test and the Clark and Lubs methyl red test.

The isolation of cultures. The cultures were all secured by plating fresh samples of feces. Twenty-one samples were used. These came from 18 subjects and furnished 176 cultures. The

samples came for the most part from men in normal health but included two stools from infants.

A small part of the sample taken at random with a platinum loop was thoroughly shaken in a water blank and plates made on asparagin-lactose-litmus agar (Ayers and Johnson 1915). These plates almost invariably gave colon colonies only and since it has been found that all types of colon bacilli grow readily on this medium while streptococci do not, it is very well adapted to isolation of these organisms from materials of this kind.

Several colonies from each sample were transferred to lactose broth and if gas was formed the broth was replated and a pure culture obtained. In a few cases special means were taken to favor the growth of certain types of colon organisms.

Pigment formation. Very few grain cultures of *B. coli* are entirely without pigment and many of them are decidedly chromogenic. This property is correlated with other characters and consequently is of value in classification.

Chromogenesis was determined in the human feces cultures by spreading on white paper the growth from an agar culture grown twelve days at 20° and comparing with the plates in Ridgway's Color Standards. It was found that this series was almost entirely lacking in pigment. Nearly all of the cultures gave a faint yellow color but this was so slight and showed so little variation that it was of no value. There were, however, a few exceptions to this statement.

Indol formation. Indol was determined by incubation at 30°C. in a medium containing in 1000 cc. of water 0.3 gram tryptophane, 5 gram K_2HPO_4 and 1 gram of Witte pepton. The test for indol was made by the p-dimethylamido-benzaldehyd-hydrochloric acid method. The results are summarized in table 3.

The liquefaction of gelatin. The test for gelatin liquefaction was made by spreading about 0.5 cc. of a twenty-four hour sugar free broth culture on gelatin held in a small test tube. This was incubated twenty days at 20°C. No gelatin liquefiers were found in this collection. This probably means that in these samples at least, liquefiers occurred in such small numbers that they were not isolated. There are numerous references in the

literature to the occurrence in feces of *B. cloacae* and *B. proteus* and it is likely that bacteria of this type would have been isolated if special methods had been employed for this purpose.

The fermentation of carbohydrates. The most convenient method for determining the extent of an acid fermentation is to measure the resulting hydrogen ion concentration (in terms of P_H). Some of the reasons for this have been treated in previous papers from this laboratory and need not be repeated here.

The procedure used was the following: The medium was composed of 1 per cent Witte pepton 0.5 per cent K_2HPO_4, 1 per cent of the carbohydrate or alcohol. This medium was distributed (in 10 cc. portions) in test tubes, sterilized by the intermittent method, inoculated from agar slopes and incubated for five days at 30°. The P_H values before and after fermentation were determined by the colorimetric procedure described by Clark and Lubs (1917a). A sufficient number of electrometric measurements were made to show that in the particular medium used the colorimetric P_H values were consistent and essentially correct.

The substances studied were glucose, lactose, sucrose, raffinose, melibiose, arabinose, inulin, mannitol, dulcitol, adonitol, and glycerol. Of these inulin and glycerol were commercial "c.p." samples. The other compounds were exceptionally pure samples furnished to us by Dr. Hudson of the Bureau of Chemistry. We take this opportunity to express publicly our appreciation of Dr. Hudson's kindness in affording us the rare opportunity to study the fermentation of sugars whose identification and purity is assured.

In the presence of most of these compounds the organisms within forty-eight hours either produce a vigorous acid fermentation or else very little change in the reaction of the medium. When a vigorous acid production occurs there is little doubt but that an acid fermentation of the carbohydrate is taking place. The converse proposition, that where no distinct acid production occurs the carbohydrate is not being utilized, is not so certain. There is a distinction here between the utilization of a substance and a particular mode of utilization which has not always been

kept clear and it is therefore necessary to emphasize the fact that we are in this paper dealing with the production of acid in carbohydrate media only as a cultural test.

It is now well established that the activity of cultures of *B. coli* is inhibited within a zone of hydrogen ion concentration near P_H 5.0 (Michaelis and Marcora, 1912; Clark, 1915). In any particular carbohydrate medium various cultures of *B. coli* arrive at a rather definite point and in media of various composition such points are generally not far apart (Clark, 1915). It has been clearly established that the concentration of the hydrogen ions is of far greater significance than the titratable acidity. But it can hardly be expected that any one definite hydrogen ion concentration would be found to characterize an organism in all media. If the hydrogen ion concentration exerts a constant influence upon the metabolism or growth of a culture the inhibition will become more pronounced as time passes so that a lower hydrogen ion concentration will be attained in a prolonged fermentation than in a rapid one. There are doubtless other substances formed which inhibit activity and the effect of these will be proportional to the time in which they act and to their concentration. There also may be present in a culture enzymatic actions proceeding independently of growth and general metabolism. Then too there are those opposing processes of acid production and destruction which the work of Ayers and Rupp (1917) has emphasized. In some cases the reaction observed at any time may be merely the resultant of these opposing processes.

The last condition is one which doubtless plays an important role in fermentations by the group of high ratio organisms. The tendency of these cultures to undergo a comparatively rapid reversion of reaction makes it difficult except under anaerobic conditions to supply enough sugar and to balance the buffer action of the medium so that the cultures can reach their limiting hydrogen ion concentrations. Apparently their limit is about the same as that of the *B. coli* or low gas ratio cultures.

All of these factors which enter into the determination of a reaction observed after any given period of time vary with the

particular sugar which is being fermented so that an analysis of the situation and the determination of those factors which are of fundamental importance in the fermentation of any given substance require more study than the mere determination of the P_H values attained in media indiscriminately put together. Studies of the fermentation of glucose have been sufficiently extended to enable Clark and Lubs (1915) to devise the so-called methyl red test for the differentiation of the high and low gas ratio groups. This test has been used in the present study and has been found to correlate perfectly with the gas ratios.

In testing the fermentation of other carbohydrates we have attached no particular significance to the P_H values but have used them merely to indicate whether a distinct acid fermentation has been dominant. It may be said, however, that the P_H values observed with similar organisms are not only fairly constant in media containing any easily fermented carbohydrate but they are obtained with much greater ease and convenience than the titration values formerly used in such tests.

In certain instances it has been difficult to decide whether the P_H values indicate a fermentation. This is particularly true of the fermentation of glycerol. The P_H of the medium changes very slowly and we suspect that in many instances there may have been a concomitant formation of alkali or destruction of acid which masked whatever acid formation may have occurred. A few examples showing how slow is the change in P_H in glycerol cultures are given in table 2. Results with sucrose, dulcitol and glycerol are assembled in figure 1. In dulcitol and sucrose broths there are two distinct modes separating the cultures sharply into fermenters and non-fermenters, but with glycerol there is only one distinct mode and that falls between the modes for dulcite and sucrose. It is evident that nearly all of these cultures ferment glycerol to some extent. Those cultures which show little or no change from the original reaction would possibly also be classed as fermenters if they were allowed sufficient time.

Notwithstanding the difficulty of making a sharp distinction in all cases between fermenters and non-fermenters by means of acidity determinations we prefer this to the gaseous fermentation

as measured by ordinary methods. We have observed numerous cases in which the reaction indicated an undoubted fermentation when so little gas was formed that it remained entirely dis-

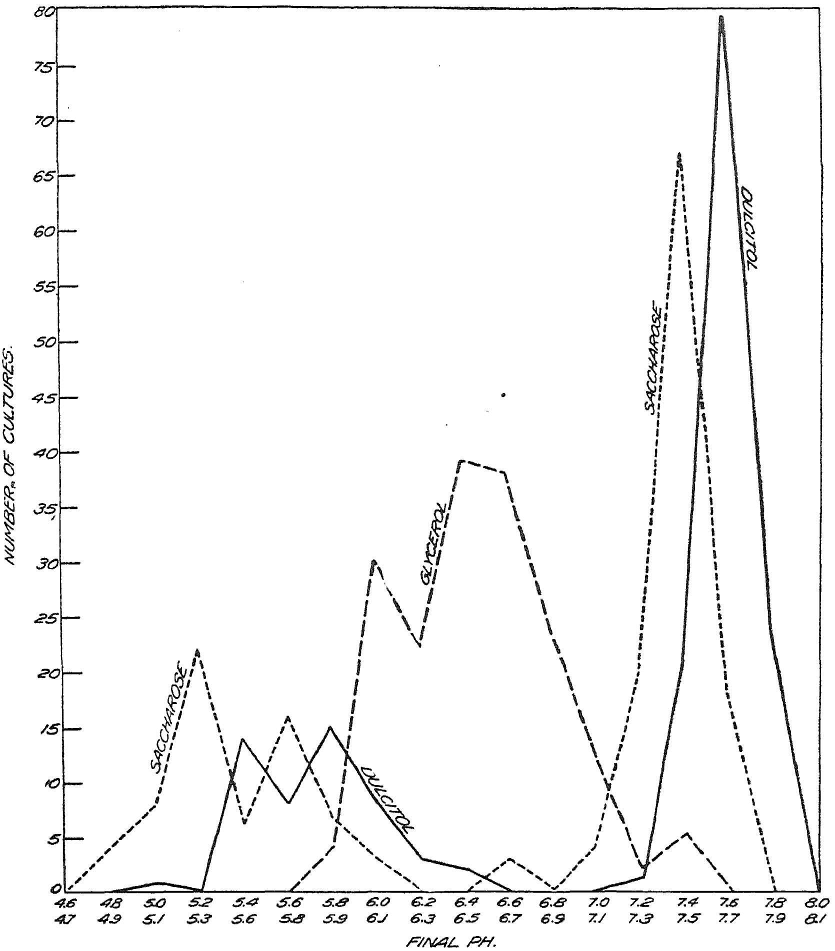

FIG 1. THE FERMENTATION OF GLYCEROL COMPARED WITH THAT OF SUCROSE AND DULCITOL

solved in the medium or appeared as a small bubble. The results of the fermentation tests are summarized in table 3.

The methyl red test. This test was made in accordance with the

procedures described by Clark and Lubs (1915) (1917b). The results given are those obtained with the old medium (Clark and Lubs, 1915). The differentiation with the new medium (Clark and Lubs, 1917b) agreed perfectly.

The Voges-Proskauer reaction. Tests for the Voges-Proskauer reaction were made with the synthetic medium of Clark and

TABLE 1

Average gas volumes and gas ratios. Low ratio cultures

SOURCE	NUMBER OF CULTURES	TOTAL GAS	RATIO CO_2/H_2
Grain	8	12.4	1.06
Bovine feces	149	14.1	1.06
Human feces	130	12.7	1.05

TABLE 2

Change in P_H *of glycerol medium by B. coli. Medium: 1 per cent Witte pepton, 0.5 per cent* K_2HPO_4*, 1 per cent glycerol. Incubation temperature, 30°. Original* P_H*, 7.6.*

ORGANISM	THREE DAYS P_H	SEVEN DAYS P_H	TEN DAYS P_H	TWENTY-ONE DAYS P_H
vk	7.6	7.6	6.7	6.3
vr	7.5	7.3	7.2	6.7
vs	7.5		7.6	6.3
vt	7.8	6.7	6.6	6.3
vo	7.5	7.4	7.2	6.5
wd	7.6	7.6	7.6	6.7
wq	7.5	7.2	6.6	6.1
wz	7.5	7.2	6.6	5.8

Lubs (1917b) and with pure casein and 10 per cent NaOH as reagents. The results summarized in table 3 are discussed by Clark and Lubs (1917b).

Gas production. In previous studies (Rogers, Clark and Davis, 1914; Rogers, Clark and Evans, 1914; Rogers, Clark and Evans, 1915) it was found that accurate determination of the gas volumes and gas ratios produced in the anaerobic fermentation of glucose furnished most valuable data. In the present study we have employed the methods for the isolation and analysis of the gas which were described in previous papers. Essentially, the

procedure consists in growing the bacteria in 10 cc. of 1 per cent Witte pepton, 0.5 per cent K_2HPO_4, 1 per cent glucose, held in evacuated sealed bulbs for seven days at 30°, collecting the gas by evacuation with mercury pumps and analyzing the gas over mercury.

The average gas volume and average gas ratio for the 131 low ratio cultures from human feces are shown in table 1 together with the averages found in the grain and bovine feces series. It

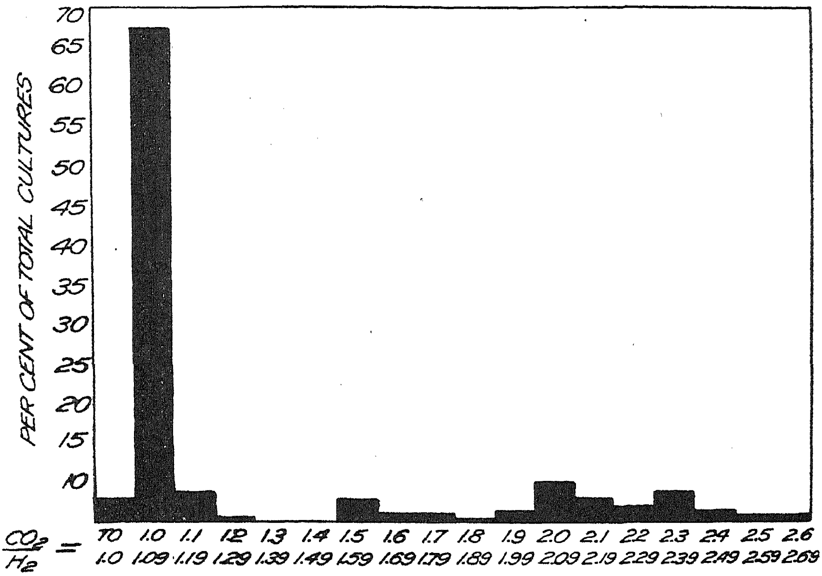

FIG. 2. DISTRIBUTION OF CULTURES ACCORDING TO CO_2 : H_2 RATIO

may be noted that the average ratio 1.06 is identical with that found by Keyes and Gillespie when their organisms were cultivated in a synthetic medium.

To bring out more clearly the sharp distinction between the high ratio and low ratio groups the distribution of cultures by gas ratios is represented graphically in figure 2.

The significant characters of the fecal cultures. It is shown in figure 2 that, on the basis of the frequency of occurrence of certain carbon dioxide-hydrogen ratios the cultures are separated into the two distinct groups already observed in cultures from milk,

from bovine feces and from grains. It should not be assumed, however, that the relative numbers of cultures as shown in this chart are indicative of the proportions in which they occur in the intestines. In bovine feces the high ratio or *B. aerogenes* group occurred so rarely that we obtained only one culture of this type in the 150 isolated. In human feces the proportion of high ratio cultures is usually greater but varies with the individual and perhaps with certain physiological conditions. In only 3 samples were *B. aerogenes* cultures obtained by direct plating. In one of these, one of seven was *B. aerogenes,* in another, 3 of 6 and in the third, of 52 colonies subjected to the methyl red test 31 were of the

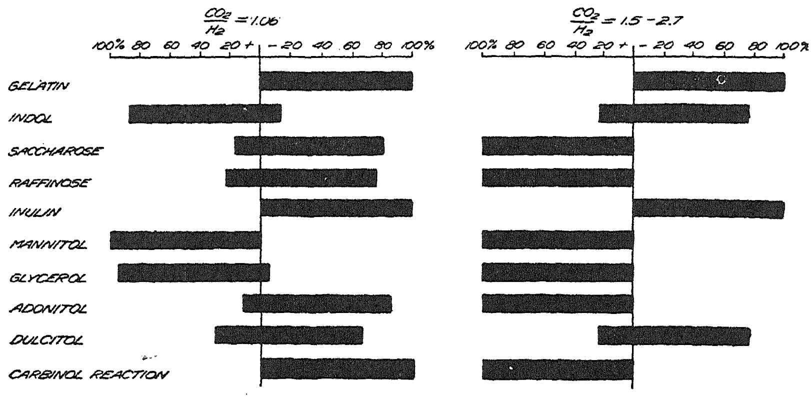

Fig. 3. Physiological Characters of the B. coli-Aerogenes Types

B. aerogenes type. All other high ratio cultures were obtained by special methods. Mr. Ayers called our attention to the fact that when sterile milk was infected with bovine feces and incubated at 20° the high ratio type predominated at the time of curdling. This holds true also for human feces and the greater part of our high ratio cultures was obtained by plating milk which had been curdled by inoculating with feces and holding at 20°C.

The predominance of the *B. aerogenes* type under these conditions is probably explained by the observation, made in the course of another investigation, that the *B. aerogenes* type is less retarded by low temperatures than is the *B. coli* or low ratio type.

The fermentation reactions of the two types of cultures are

TABLE 3

Showing percentage of positive physiological reactions

	TOTAL CULTURES		INDOL	CARBINOL REACTION	SUCROSE	RAFFINOSE	INULIN	MANNITOL	GLYCEROL	DULCITOL	ADONITOL
Low ratio...	131	Number +	127	0	23	31	0	130	124	41	17
		Per cent +	96.95	0	17.56	23.66	0	99.23	94.66	31.78	12.98
High ratio...	46	Number +	10	46	46	46	0	46	46	10	46
		Per cent +	21.74	100	100	100	0	100	100	21.74	100

TABLE 4

Per cent of positive reactions obtained with cultures from various sources

GROUP	SOURCE OF CULTURES	NUMBER OF CULTURES	INDOL	SUCROSE	RAFFINOSE	INULIN	MANNITOL	GLYCEROL	ADONITOL	DULCITOL
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Low ratio (coli)	Milk	58	87.90	39.70	43.10			56.90	15.80	50.00
	Bovine feces	149	99.30	59.10	62.40	0	100.00	97.30	20.80	68.20
	Human feces	131	96.95	17.56	23.66	0	99.23	94.66	12.98	31.78
	Grains	8	50.00	25.00	75.00	12.5	100.00	25.00	37.50	50.00
	Feces*	306	98.02	46.08		0			9.48	74.18
	Feces†	117	95.72	41.88	51.28			75.82		53.85
High ratio (aerogenes).	Milk	56	60.70	96.40	91.00			18.50		26.80
	Human feces	46	21.74	100.00	100.00	0	100.00	100.00	100.00	21.74
	Grain	111	7.20	88.29	85.58	10.81	20.72	56.76	12.61	16.21
	Feces*	11	0	100.00		0			100.00	27.27

* MacConkey, 1909. MacConkey's results are recalculated assuming that all V and P positive cultures are *B. aerogenes* and V and P negative cultures are *B. coli*.

† Levine (1916). Includes cultures from horse, sheep, cow and man.

shown in table 3 and figure 3. The correlation between the nature of the fermentation as expressed by the resulting gases and the ability to produce acid from certain substances agrees very closely with that which we have found in the colon cultures from other sources and with the correlations found by other investigators.

This agreement is shown in table 4 which includes our own

data, that of some 300 cultures whose characters have been tabulated by MacConkey and the data of 117 cultures reported by Levine. The results obtained by Levine show that it is safe to separate MacConkey's cultures into the high and low-ratio groups by means of the Voges and Proskauer test. If we exclude the eight grain cultures, the agreement among the low ratio cultures is as close as could be expected after making proper allowance for differences in methods. The relatively low percentage of indol formers among the milk cultures is probably due to the use of a method which was later found to give low results.

It will be noted that there was an appreciably higher number

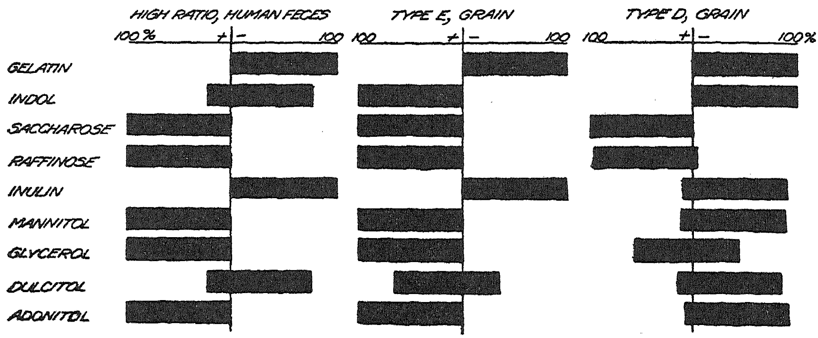

FIG. 4. A COMPARISON OF THE HIGH RATIO CULTURES FROM FECES WITH TWO TYPES FROM GRAINS

of cultures fermenting sucrose, raffinose and dulcitol among the bovine feces cultures than among those from human feces. MacConkey's cultures, which in this respect are intermediate between the human and bovine cultures, include 138 from feces of various animals.

While Levine's results as to the relative number of sucrose fermenters in feces from various animals are not conclusive on account of the comparatively small number from each source, he found a decidedly higher percentage of sucrose and raffinose fermenters among the cultures from the lower animals than among those from man. The percentages for sucrose fermentation ranged from 32.3 per cent for cultures from pigs to 95.5 per cent for the sheep as against only 12 per cent for man.

Browne (1915) found that about 11 per cent of colon cultures from human stools fermented sucrose while about 40 per cent fermented dulcitol. These results are in fairly close agreement with our own.

When we turn to the high ratio or *B. aerogenes* group we find a more complex situation. The non-liquefying high-ratio grain cultures were easily separable into three distinct types. For purposes of comparison two of these are arranged with the high ratio fecal group in figure 4. Type E, in which were included only 8 cultures, presents a picture almost identical with that shown by the diagram for the high ratio fecal group. There was a higher percentage of indol formers among the grain cultures and a slightly larger proportion of dulcitol fermenters; otherwise the agreement is perfect and it may be assumed that the 8 grain cultures were originally of fecal origin.

The diagram for type D, which included 90 cultures, shows the typical *B. aerogenes* culture found on grains. While it agrees with the high ratio human feces cultures in certain characteristics it is sharply differentiated from them by its feeble action on the alcohols, particularly on adonitol. The *B. aerogenes* cultures from feces without exception ferment this alcohol readily while with the exception of those classified as of fecal origin only 6 of 103 of the *B. aerogenes* cultures from grains were able to utilize this substance.

These results indicate that there is reason for separating the high ratio type found in feces from the high ratio type found on grain as shown by type D in figure 4. The difference in ability to utilize adonitol is distinctive and if the separation is a logical one it should be of value to water bacteriologists.

In a previous paper (Rogers, Clark and Davis, 1914) it was shown that the low gas ratio if not modified by oxidations of the hydrogen remains constant under a variety of conditions which permit wide variations in the high gas ratios. This together with other considerations led to the conclusion that the carbon dioxid and the hydrogen liberated by *B. coli* are intimately connected. The establishment of so constant and simple a ratio between two products furnishes a most reliable sort of cultural characteristic

and justifies our setting apart the low ratio organisms as distinct from the more heterogeneous collection of high ratio organisms. Whether or not this will ultimately prove to be sound judgment the fact remains that it has been of great empirical value. But we have not been content with using this basis of classification alone. If it is a proper basis for a primary classification, there should appear decided correlations between the gas ratios and other cultural tests. As our studies have progressed, evidences of such correlations have become stronger and are becoming more clearly established as various tests become more rational and

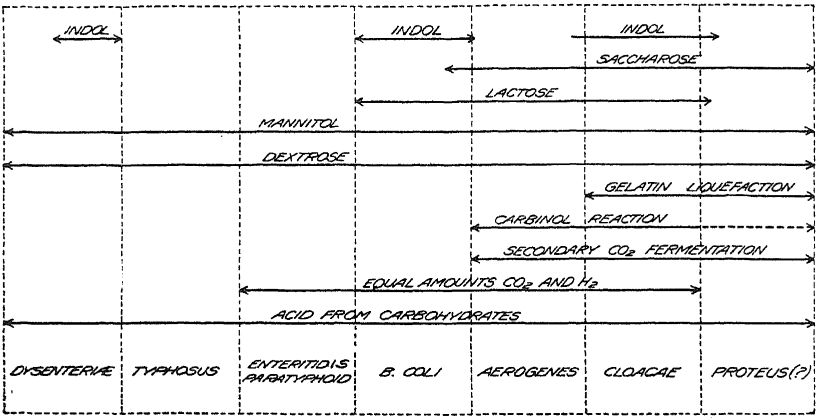

FIG. 5. GRAPHIC REPRESENTATION OF THE SALIENT CHARACTERS OF THE TYPHOID COLON, AEROGENES GROUP

exact. Hulton (1916) in discussing our results says: "In their system of classification, the gas ratio formed the only basis of differentiation; members classed within a single group display marked differences in powers of liquefaction, indol-production, and motility, but all agree in gas ratios." In view of the fact that tests of indol production, gelatin liquefaction and motility have not yet been placed upon an exact basis and because the gas ratios correlate perfectly with the methyl red and Voges-Proskauer tests in the present series, we should perhaps be willing to have it understood that the gas ratios formed the only basis of primary classification. This, however, would hardly be a correct

statement of our views since we have shown by biometric treatment correlations among other cultural characteristics which are quite as good as those obtained in other schemes of classification.

Kligler (1914), as the result of a study of 80 cultures in the collection of the American Museum of Natural History, proposed a classification based upon the ability to ferment certain sugars and alcohols. This was a step in advance in that it was based on correlated characters. We are not ready to admit, however, that the fermentation of polysaccharides is in itself a sufficiently fundamental character to be used for major divisions of a large group.

Levine (1916b) has proposed a classification of the colon-aerogenes group in which particular attention is given to those cultures which are included in figure 5 as *B. coli*. Six species are made of this group, one of which includes two varieties. The separations are made on sucrose fermentation, motility and the fermentation of salicin, dulcitol and glycerol. We have always considered that *B. coli* as represented in figure 5 is comparatively a homogenous group and that the differences indicated by variations in sugar fermentations are not of specific rank. On the other hand, we have looked upon *B. aerogenes*, which Levine includes as a single species, as a heterogenous group much in need of further division.

The greatest value of definite knowledge of the characteristics of a class of organisms lies in the ability which it gives us to arrange these organisms in orderly groups. Ever since the beginning of the science bacteriologists have attempted to do this and as the knowledge of bacteria has advanced new systems of classification have been proposed, many of them differing radically from their predecessors.

The cumulative result of these attempts to create order has brought such confusion that we have hesitated to apply our results to any system of classification for the colon-aerogenes group. We feel, however, that the data which have accumulated in the last few years have sufficient value to warrant us in outlining their possible application to taxonomic problems. While there is no generally accepted scheme of classification for the typhoid-colon

aerogenes group there are certain salient points which nearly all bacteriologists will agree may be accepted as separating the group into rather loosely defined subgroups or species. These may be outlined briefly as follows:

Characters common to the group—short, thick, gram negative bacillus, tendency to ferment carbohydrates to some degree; normal habitat, intestines of warm-blooded animals

Dysenteric group	Acid from glucose and sometimes from mannitol and sucrose. Indol, ±. Carbinol reaction—Characteristic serologic and pathogenic properties.
Typhoid group	Acid from glucose and mannitol. Indol and carbinol reaction –. Characteristic serologic and pathogenic properties.
Paratyphoid, enteritidis group	Acid and gas from glucose, mannitol and dulcitol. Indol and carbinol reaction negative. Characteristic serologic and pathogenic properties.
B. coli group	Acid and gas from glucose, lactose, mannitol and sometimes sucrose and dulcitol. Volume of gas relatively small. Indol usually positive, carbinol reaction usually negative.
B. aerogenes group	Acid and gas from glucose, lactose, sucrose and usually from mannitol, but not from dulcitol. Volume of gas greater than *B. coli*. Indol – and carbinol reaction ±.
B. cloacae group	Characters very similar to aerogenes and in addition gelatin is liquefied.
Proteus group	Liquefies gelatin and under certain conditions forms characteristic "swarming" colonies. Acid and gas from glucose but lactose is not fermented.

A more definite classification of the colon-aerogenes group is offered by the MacConkey arrangement which was adopted by the laboratory section of the American Public Health Association. This is purely an arbitrary classification based on the possible mathematical combinations of positive and negative reactions obtained with dulcitol and sucrose. It has no logical basis or practical value.

The data concerning the fundamental characteristics of the several groups in this large family of bacteria is without doubt too meagre at present for the establishment of a system of classifica-

tion which will have permanent value. There is, however, some justification in proposing a scheme of classification if it possesses those elements of plausibility and suggestiveness which render working hypotheses valuable assets in research. We now suggest such a scheme which is based primarily upon a hypothesis proposed in the first paper of this series (Rogers, Clark and Davis, 1914).

The internal mechanism by which an organism converts its food into energy and cell material is conceived to be more deep seated and less variable than the processes by which a particular substance is prepared for utilization. It is generally assumed that substances like sucrose must be hydrolized before the constituent glucose or fructose can be utilized. While definite evidence of this is lacking we may assume it to be true. It is then conceivable that since the inverting enzyme has only a preparatory function it may be subject to some variation as the food supply of the bacteria changes while the processes through which the ultimate utilization of the carbohydrate is brought about remain constant. The nature of the inverting enzymes is shown by the particular polysaccharides utilized while the processes which produce the final decomposition are indicated by the end products of the fermentation.

Such a conception assigns a position of secondary importance to certain sugar fermentations and leads to a search for the significant aspects in the ultimate metabolism. In the colon-aerogenes group an indication of one special mechanism of fermentation is found in the varying amount and nature of the gases produced.

As previously suggested (Rogers, Clark and Davis, 1914) the reaction by which carbon dioxid and hydrogen are liberated in equal volumes may be common to the high and low ratio organisms and the high gas ratio may result from an additional reaction in which carbon dioxid alone is set free. It is suggestive to note that we now have one group which produces equal volumes of carbon dioxid and hydrogen, another which produces an excess of carbon dioxid and a third which produces carbon dioxid only and that all three groups appear to be generically

related in so far as a general similarity in their morphology, relation to external conditions and their metabolic activities betokens such a relationship.

Upon such a basis, which is of course hypothetical, there can be constructed the provisional scheme of classification shown in figure 5. This scheme recognizes in all members of the group an ability to bring about some sort of fermentation of the simplest carbohydrates. In what may be considered the lower members of the group this fermentation is limited to the formation of acids from simple sugars such as glucose. The four higher groups beginning with *B. enteritidis* possess the ability to institute a fermentation in which carbon dioxide and hydrogen are formed in equal volumes. The *B. aerogenes* and *B. cloacae* groups are differentiated still further by a secondary fermentation producing additional carbon dioxide. With this is correlated the somewhat obscure carbinol reaction and in the case of *B. cloacae* the secretion of proteolytic enzymes. The position of the so-called *B. proteus* group in this diagram is questionable. We have designated as *B. proteus* those organisms which produce no hydrogen. This is not compatible with the characteristics of several cultures which others have furnished to us as *B. proteus* but it is based on data obtained with some of these *B. proteus* cultures from other laboratories and on data obtained with seven cultures in our collection from grain. All of these seven cultures liquefied gelatin and six failed to ferment lactose. They would ordinarily have been classed as *B. proteus*, but whatever name is used, such a group exists and is of relatively common occurrence.

Inasmuch as all of our *B. proteus* cultures ferment sucrose they have the general characteristics which would be possessed by a *B. cloacae* culture in which the fermentation producing equal volumes of carbon dioxide and hydrogen had been suppressed.

This diagram, we believe, represents the probable evolution from the highly organized *B. cloacae* group, amply qualified to live an independent existence, to the parasitic pathogenic types. This hypothesis is in accord with the reported distribution of the different types. *B. cloacae* is common in soil and water while *B. aerogenes*, its close relative, was found by us very commonly

on grains and in water but only occasionally in feces. *B. coli*, on the other hand, is the principal organism found in the intestines and does not thrive in natural conditions outside of the animal body. All of the evidence available indicates that the enteritidis, typhoid and dysenteric groups do not live normally outside of the animal body. The proteus group is possibly an offshoot of *B. cloacae* in which a part of the fermentative ability has been lost.

No biological species or genus has sharply defined limits and it should not be assumed that the physiological characters are restricted in the precise manner indicated in this diagram. It is commonly assumed, for instance, that all members of the colon-aerogenes group ferment lactose but it is very improbable that a sharp distinction could be drawn in this way without excluding cultures which by all other characters belong in this category. While this distinction is habitually made in water bacteriology, it is generally recognized that the exclusion of cultures, which, while otherwise resembling colon, do not ferment lactose, is a concession to the demand for rapid results justified by the fact that it does not introduce a serious error.

Clemesha (1912) speaks of a colon organism fermenting glucose but not lactose as making up 4 or 5 per cent of the flora of the intestines and says that lakes in India exposed for some time to sunlight contained more of this type than any other. Our own collection from human feces was made on the basis of fermentation of lactose in conformity with the usual practice of American sanitarians. It included, however, one culture which fermented glucose but not lactose. This did not differ otherwise from the typical low ratio culture.

It may be argued that lactose is a unique carbohydrate in that it is distinctly an animal sugar and that its fermentation or non-fermentation is of more fundamental significance than the fermentation of a distinctly plant sugar. We fail to see any logic whatever in such an argument. It is comparable with connecting fermentability with optical rotation, neglecting the more weighty matters of configuration and linkage within the sugar group. It may be significant to note that of 177 cultures which

we have studied in the present series all but one ferment both lactose, a sugar of animal origin, and melibiose, a plant sugar closely related to lactose. The one culture which failed to ferment lactose failed to ferment melibiose.

In this scheme of classification the positions assigned to the lower groups are determined in large measure by the gas formation. While the data found in the literature are for the most part unreliable and the results which we have obtained are meagre, the following determinations are significant.

Cultures of *B. paratyphi*, *B. enteritidis* and *B. dysenteriae* from the American Museum were grown at 37°C. on the medium used in the gas determinations with colon cultures and the gas volume and ratios were determined by the accurate method previously described.

The results are as follows.

ORGANISM	TOTAL GAS	RATIO CO_2/H_2
B. paratyphi "A." American Museum no. 294	7.09	1.00
B. paratyphi "A." American Museum no. 16	13.83	1.03
B. paratyphi "B." American Museum no. 323	8.60	1.06
B. paratyphi "B." American Museum no. 22	10.08	1.01
B. enteritidis American Museum no. 18	12.24	1.03
B. dysenteriae (Kruse) American Museum no. 121	Trace	Not analyzed
B. dysenteriae (Shiga) American Museum no. 197	Trace	Not analyzed
B. dysenteriae (Flexner) American Museum no. 110	Trace	Not analyzed

Keyes and Gillespie (1912) have reported hydrogen and carbon dioxide as products of the activity of *B. typhi*. This might be considered to invalidate the position given to *B. typhi* in figure 5. However, Keyes and Gillespie obtained only about 0.2 cc. total gas per 10 cc. with *B. typhi* and the gas ratio CO_2/H_2 was large and inconstant.

Theoretically it is of course unnecessary to assume that carbon dioxide is a constant product of the metabolism of bacteria, yet experimentally there are evidences that small amounts of this gas are formed by certain "non-gas producing" bacteria. This is neglected in figure 5 as an unessential consideration.

SUMMARY

A collection of 177 cultures of the colon-aerogenes type was made from human feces.

These were examined for pigment formation, indol production, liquefaction of gelatin, the acid fermentation of glucose, lactose, sucrose, raffinose, melibiose, arabinose, inulin, mannitol, dulcitol, adonitol, and glycerol, the methyl red test, the carbinol or Voges-Proskauer reaction and the anaerobic production of gas from glucose as determined by exact methods.

Of the 177 cultures studied, 131 gave a CO_2/H_2 ratio of approximately 1.06. The remaining 46 cultures gave ratios varying from 1.5 to 2.7.

The methyl red tests correlated perfectly with the gas ratios.

None of these cultures fermented inulin but practically all fermented mannitol and glycerol. Nearly all of the low ratio cultures formed indol but all were negative to the carbinol reacaction. A relatively small percentage fermented sucrose, raffinose, dulcitol and adonitol.

The high ratio cultures included a small percentage of indol producers but all gave a positive carbinol reaction. All of them fermented sucrose, raffinose, and adonitol. Only a small number fermented dulcitol.

The high ratio group agreed very closely with a group represented by a small number of cultures isolated from grains and described in an earlier paper. The greater number of the grain cultures differed from the fecal cultures in their ability to ferment alcohols, particularly adonitol.

A tentative scheme of classification for the typhoid colon group is presented in which the primary divisions are based on the nature of the metabolism as indicated by the gases evolved.

The gas analysis of a few available cultures of the paratyphoid group gave a gas volume and ratio agreeing with that of the colon type.

REFERENCES

AYERS AND JOHNSON 1915 A bacteriological study of retail ice cream. U. S. Dept. of Agric. Bull. 303.

Ayers and Rupp 1917 In press.
Browne, W. W. 1915 J. Infect. Dis., **17**, 72-78.
Clemesha, W. W. 1912 The bacteriology of surface waters in the tropics. London, 1912.
Clark 1915 J. Biol. Chem., **22**, 87.
Clark and Lubs 1915 J. Infect. Dis., **17**, 160.
Clark and Lubs 1917a J. Bact., **2**, 1–34, 109–136, 191–236.
Clark and Lubs J. Biol. Chem. 1917b, 30, 209.
Holton 1916 J. Infect. Dis., 19, 606.
Keyes 1909. J. Med. Research, **21**, 69.
Keyes and Gillespie 1912 J. Biol. Chem., **13**, 291.
Kligler, I. J. 1914 J. Infect. Dis. **15**, 186–204.
Levine, M. 1916a J. Infect. Dis., **19**, 773-805.
Levine, M. 1916b J. Bact., **1**, 619.
MacConkey, A. 1909 J. Hyg., **9**, 86.
Michaelis and Marcora 1912 Z. Immunitätsforsch. Exper. Ther. (orig.) **14**, 170.
Rogers, Clark and Davis 1914 J. Infect. Dis., **14**, 411–475.
Rogers, Clark and Evans 1914 J. Infect. Dis., **15**, 100–123.
Rogers, Clark and Evans 1915 J. Infect. Dis. 17, 137–159.

A STATISTICAL CLASSIFICATION OF THE COLON-CLOACAE GROUP

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It is now firmly established that the end products of metabolism, as well as morphological differences, are reliable and convenient indices for differentiation of bacterial species and varieties. In the group of coli-like bacteria, particular attention has been paid to acid and gas production fron various fermentable substances.

Theobald Smith (1893) observed that some *B. coli* ferment sucrose and therefore recognized two forms.

Durham (1901) suggested the name *B. coli-communior* for the sucrose fermenting variety and characterized *B. lactis-aerogenes* as a polysaccharid fractor.

MacConkey (1905 and 1909), whose classification has been most widely employed, subdivided upon gas formation from sucrose and then from dulcitol thus giving 4 main types generally known as the *B. acidi-lactici* type (sucrose negative, dulcitol negative); the *B. communis* type (sucrose negative dulcitol positive); the *B. communior* type (sucrose positive, dulcitol positive), and the *B. aerogenes* type (sucrose positive, dulcitol negative). Under each type are recorded a number of varieties according to gelatin liquefaction, indol production, the Voges-Proskauer reaction, motility, and fermentation of inulin, adonitol, etc.

Very much along the lines of the MacConkey classification is that of Bergey and Deehan (1908). They employed 8 characters —fermentation of sucrose, dulcitol, adonitol, and inulin; gelatin liquefaction, indol production, motility and Voges-Proskauer reaction—and from a consideration of all possible combinations

between these characters recognized the possible existence of 256 varieties of *B. coli*.

The grouping of Jackson (1911) which was accepted by the American Public Health Association and included in the standard methods for 1912, is very similar to that of MacConkey, but here preference is given to dulcitol over sucrose for the primary division. Each of the 4 groups thus formed is subdivided further on raffinose and mannitol, and then on motility, indol, reduction of nitrates, and gelatin liquefaction.

A very serious objection to the classifications of MacConkey, Bergey and Deehan, and Jackson, is their extreme flexibility. As the number of fermentable substances, or other characters observed, increases, the number of "varieties" increases geometrically approaching infinity. The number of "varieties" is given by the formula 2^n where 'n' is the number of characters studied. Thus with 8 characters there are 256 possible combinations; this number rises to 1024 with 10 characters and to 65,536 when 16 characters are observed. The absurdity of regarding each character as of similar and equal differential value is thus evident. In the more recent studies the principle of the correlation of characters has been emphasized.

Howe (1912), from a statistical study of 630 strains of *B. coli* isolated from human feces, concludes that dulcitol, indol production, nitrate reduction, etc., are not correlated with each other nor with vigor of growth, and he therefore recognizes only the sucrose positive *B. communior* and sucrose negative *B. communis*.

Rogers and his associates, (1914–1916) studied a large number of coli-like forms from milk, grains, and bovine feces, and conclude that two distinct groups may be recognized on the basis of the accurately determined gas ratio—the low ratio *B. communis-B. communior* group and the high ratio *B. aerogenes-B. acidi-lactici* group. There is no doubt that *B. communis* and *B. communior* are low ratio strains and *B. aerogenes* of the high ratio group but the inclusion of *B. acidi-lactici* with *B. aerogenes* does not seem justified, and I believe that further studies will place it definitely with the low ratio strains.

Kligler (1915) suggests that salicin be substituted for dulcitol, in subdividing coli-like bacteria, pointing out that salicin fermentation correlates better with the Voges-Proskauer reaction than does dulcitol decomposition. He thus recognizes a sucrose negative, salicin negative group (*B. acid-lactici*); sucrose negative, salicin positive group (*B. communis*); sucrose positive, salicin negative group (*B. communior*) and sucrose positive, salicin positive (*B. aerogenes*). *B. cloacae* is differentiated from *B. aerogenes* by its inability to ferment glycerol.

The characterization of *B. communior* as salicin negative is probably untenable. The term *B. coli-communior* was first employed by Durham to describe a variety of *B. coli* which fermented sucrose and which was motile. Later Ford recognized it as a species *B. communior.* Such organisms usually ferment salicin as will be shown later in this paper.

Where the principle of correlation has been employed the best correlated character has apparently been picked out by inspection of the data. Inspection is a tedious and difficult procedure, entirely inapplicable where the number of characters considered is large, and it does not permit of a concise statement of the degree of correlation which exists between different reactions. Considerable information in an abstract, concise, and workable, form may however be obtained from a study of the coefficients of correlation.

THE COEFFICIENT OF CORRELATION

Where we are concerned merely with the presence or absence of characters the coefficient of correlation between any two characters may be easily determined. Suppose that it is desired to know if the characters X and Y are correlated and that a study of a number of organisms showed that 'a' cultures are positive for both X and Y; 'b' organisms positive for X but negative for Y, 'c' cultures are negative for X and positive for Y; and 'd' strains are negative for both X and Y. The distribution of the organisms is first tabulated as shown below.

		Y +	Y −
X	+	a	b
	−	c	d

The degree of association, or the coefficient of correlation, is then expressed, according to Yule, by the formula

$$\frac{ad - bc}{ad + bc} \tag{1}$$

If 'ad' is equal to 'bc' the coefficient becomes $\frac{0}{ad + bc}$ or 0; which indicates that there is no correlation whatever. If either 'b' or 'c' is zero the formula becomes $\frac{ad}{ad} = 1$; indicating a perfect positive correlation. If 'a' or 'd' is zero then we have $\frac{-bc}{bc} = -1$; showing a perfect negative correlation. It should be observed that an absolute positive correlation exists in reality only if both 'b' and 'c' are zero and an absolute negative correlation when both 'a' and 'd' are zero. In order to avoid coefficients of 1 or -1 where only one group—'a', 'b,' 'c,' or 'd'—is zero. Yule gives the formula

$$\frac{a\ (a+b+c+d) - (a+c)\ (a+b)}{\sqrt{(a+c)\ (b+d)\ (a+b)\ (c+d)}} \tag{2}$$

In practice, however, a few strains are almost always found in each of the four groups and Yule suggests the use of the simpler formula (1). Some caution should therefore be employed in interpreting coefficients of 1 or -1.

For this study it was assumed that if the coefficient between two characters is numerically greater than 0.5 they may be regarded as correlated, but if less than 0.3 there is probably no association. A few examples of correlation coefficients actually obtained in the course of this study are given to illustrate the method of calculation.

M.R. \ V-P	+	−
+	7	43
−	132	2

$$\frac{7\times 2 - 43\times 132}{7\times 2 + 43\times 132} = -1.00$$

Negative Correl'n

Raffinose \ Sucrose	+	−
+	89	7
−	4	82

$$\frac{89\times 82 - 4\times 7}{89\times 82 + 4\times 7} = +.99$$

Positive Correl'n

Dulcitol \ Salicin	+	−
+	75	22
−	39	46

$$\frac{75\times 46 - 22\times 39}{75\times 46 + 22\times 39} = +.61$$

Partial Correl'n

Dulcitol \ Indol	+	−
+	87	10
−	76	9

$$\frac{87\times 9 - 76\times 10}{87\times 9 + 76\times 10} = +.02$$

No Correlation

The principle of correlation should not be applied indiscriminately to collections of data for systematic purposes. Certain characters and properties have been universally accepted as reliable and appropriate for bacterial differentiation; thus, staining reactions such as the Gram and acid fast stains; spore formation, aerobiosis and anaerobiosis, hardly need to be bolstered up by correlation with other characters to justify their taxonomic value. On the other hand the significance of such characters as motility, indol production, and fermentation of certain substances, is still debatable.

Motility is regarded by many as a highly variable property. Perhaps it is in reality a reliable morphological difference. Certainly if it could be shown that this character goes hand in hand with several others, more reliance and attention should and would be given to motility. The same is true of the indol test. In dealing with gas formation from carbohydrates, alcohols, or polysaccharids, the question naturally arises as to which substance should be given preference for subdivision, or whether all are to be considered of equal taxonomic value. The lack of a criterion for determining the most significant fermentable substances has led to considerable confusion. It has already been pointed out how subdivision on every character studied results in an infinite number of varieties. Where we are dealing with a number of characters each of which is assumed to be of equal taxonomic significance, it would certainly be desirable and advantageous to subdivide on that character which gives the greatest amount of information as to the manner in which the resulting subgroups react with respect to other characters. It is under such circumstances that the principle of correlation of characters may be legitimately, conveniently, and advantage-

ously employed. It may be recalled that the differentiation of the colon-intermediate-typhoid group on glucose and lactose fermentation is strikingly correlated with pathogenicity.

STATISTICAL STUDY

In the following pages is evolved a classification of coli-like bacteria based primarily upon correlated characters. The study is made upon 333 organisms obtained from soil, sewage and the feces of man, horse, sheep, pig and cow.

The characters considered are the methyl-red and Voges-Proskauer reactions, indol production, motility, gelatin liquefaction and gas formation from sucrose, raffinose, dulcitol, glycerol, salicin, dextrin, inulin and corn starch. Other fermentable substances—lactose, maltose, galactose and mannitol—were also observed but as these substances were all attacked with gas formation they need not be considered.

The investigations of Theobald Smith, Hardin, Rogers and others indicate distinctly that the Voges-Proskauer positive or methyl-red negative strains are so different from the Voges-Proskauer negative, or methyl-red positive organisms with respect to the end products of carbohydrate fermentation that subdivision upon these characters seems justified. Two groups are therefore recognized, the methyl-red positive, Voges-Proskauer negative or *B. coli group* and the methyl-red negative, Voges-Proskauer positive or *B. aerogenes-B. cloacae* group.

METHOD OF STUDY

The organisms in each of the two groups are first tabulated as in table 1 in order to facilitate the calculation of the correlation coefficients which are then determined for each pair of characters and recorded as indicated in table 1A. In choosing between any two characters, that one which gives the highest coefficient of correlation with the greatest number of other characters is selected for subdivision. For the resulting subgroups new correlation tables are prepared and subdivision again made as above. A point is very quickly reached where further subdivision upon

correlated characters is no longer feasible. These groups are regarded as species and to each is assigned, as far as possible, the name of the MacConkey variety which it most resembles.

TABLE 1

Showing correlation of characters among 151 strains of the Aerogenes-cloacae group

		Gelatin		Motility		Indol		Sucrose		Raffinose		Dulcitol		Glycerol		Salicin		Dextrin		Inulin*		Starch	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Gelatin	+	83		81	2	13	70	83		79	4	11	72	7	76	82	1	27	56	4	79	5	78
	−		68	8	60	33	35	65	3	66	2	34	34	62	6	67	1	63	5	18	41	60	8
Motility	+	81	8	89		15	74	86	3	83	6	14	75	8	81	87	2	28	61	4	84	4	85
	−	2	60		62	31	31	62		62		31	31	61	1	62		62		18	36	61	1
Indol	+	13	33	15	31	46		46		46		24	22	37	9	46		39	7	9	31	33	13
	−	70	35	74	31		105	102	3	99	6	21	84	32	73	103	2	51	54	13	89	32	73
Sucrose	+	83	65	86	62	46	102	148		144	4	44	104	69	79	146	2	90	58	22	117	65	83
	−		3	3			3		3	1	2	1	2		3	3			3		3		3
Raffinose	+	79	66	83	62	46	99	144	1	145		44	101	68	77	143	2	88	57	21	115	65	80
	−	4	2	6			6	4	2		6	1	5	1	5	6		2	4	1	5		6
Dulcitol	+	11	34	14	31	24	21	44	1	44	1	45		38	7	45		37	8	18	22	33	12
	−	72	34	75	31	22	84	104	2	101	5		106	31	75	104	2	53	53	11	89	32	74
Glycerol	+	7	62	8	61	37	32	69		68	1	38	31	69		69		66	3	20	41	63	6
	−	76	6	81	1	9	73	79	3	77	5	7	75		82	80	2	24	58	2	79	2	80
Salicin	+	82	67	87	62	46	103	146	3	143	6	45	104	69	80	149		89	60	22	119	65	84
	−	1	1	2			2	2		2			2		2		2	1	1		1		2
Dextrin	+	27	63	28	62	39	51	90		88	2	37	53	66	24	89	1	90		22	60	65	25
	−	56	5	61		7	54	58	3	57	4	8	53	3	58	60	1		61		60		61
Inulin*	+	4	18	4	18	9	13	22		21	1	10	11	20	2	22		22		22		21	1
	−	79	41	84	36	31	89	117	3	115	5	22	89	41	79	119	1	60	60		120	36	84
Starch	+	5	60	4	61	33	32	65		65		33	32	63	2	65		65		21	36	65	
	−	78	8	85	1	13	73	83	3	80	6	12	74	6	80	84	2	25	61	1	84		86

* Nine strains not tested in inulin.

THE AEROGENES-CLOACAE GROUP

In the *B. aerogenes-B. cloacae* group are included all strains which gave the Voges-Proskauer reaction—(practically always alkaline to methyl-red) and 10 cultures which fermented starch with gas formation but did not react typically for the Voges-Proskauer nor methyl-red tests. There are 151 organisms in the group, 9 of which were obtained from sewage and the rest, 142, from soil.

The distributions of the strains with respect to gelatin liquefaction, motility, indol and gas formation from sucrose, raffinose, dulcitol, glycerol, salicin, dextrin, inulin and starch are shown in table 1. Mannitol, maltose, lactose and galactose were always fermented and are therefore not included.

Gelatin was liquefied by 83 (55 per cent) (observed for thirty-four days at 20°C.); 89 (59 per cent) were motile; 46 (30.5 per cent) formed indol from Witte's peptone, and gas was formed as follows: sucrose 148 (98.2 per cent); raffinose 145 (96.2 per cent); dulcitol 45 (29.8 per cent); glycerol 69 (45.6 per cent); salicin 149 (98.8 per cent); dextrin 90 (59.5 per cent); inulin 22 (14.6 per cent) and starch 65 (43 per cent). It is evident from table 1 that sucrose, raffinose and salicin, because of their extreme availability, cannot be employed for differentiation within the group. The coefficients of correlation for each pair of remaining characters are given in table 1A.

TABLE 1A

Coefficients of correlation for each pair of characters in table 1

	Gelatin	Motility	Indol	Dulcitol	Glycerol	Dextrin	Inulin	Starch
Gelatin		+.99	-.67	-.74	-.98	-.93	-.79	-.98
Motility	+.99		-.66	-.69	-.99	-1.00	-.83	-1.00
Indol	-67	-.66		+.63	+.80	+.71	+.33	+.71
Dulcitol	-.74	-.69	+63		+.86	+.65	+.62	+.73
Glycerol	-.98	-99	+.80	+.86		+.97	+.90	+.99
Dextrin	-.93	-1.00	+.71	+.65	+.97		+1.00	+1.00
Inulin	-.79	-.83	+.33	+.62	+.90	+1.00		+.96
Starch	-.98	-1.00	+.71	+.73	+.99	+1.00	+.96	

Mere inspection of table 1A shows that gelatin liquefaction is almost perfectly correlated with motility and fermentation of glycerol, dextrin, and starch; the association being positive with motility and negative with the others. Similarly motility is

correlated with glycerol, dextrin, starch and gelatin. Each of these characters is correlated with each other. Under these circumstances any of these reactions may be selected for subdivision; the choice depending upon which were employed in an investigation and to some extent on the personal preference of the investigator. The characterization of *B. aerogenes* by Durham as a starch fermenter; the differentiation of *B. aerogenes* from *B. cloacae* by MacConkey on gelatin liquefaction and motility, and by Kligler on glycerol fermentation are all correct; the apparent confusion being the inevitable result of separation upon single characters.

Two species are evidently present, the *B. aerogenes* which rarely, if ever, liquefies gelatin; is non-motile; and forms gas from glycerol and starch; and the *B. cloacae* which liquefies gelatin (often very slowly); is motile; and does not form gas from glycerol nor starch. As gelatin liquefaction is an inconvenient character the organisms are subdivided for further study upon motility into the non-motile *B. aerogenes* and the motile *B. cloacae*. Glycerol or starch would do just as well. Whichever character is selected, a few strains are present in each of the resulting groups which possess some of the salient characteristics of the other. Thus of 89 motile strains 8 did not liquefy gelatin, 8 formed gas from glycerol and 4 from starch, while of 62 non-motile strains, 2 liquefied gelatin, and glycerol and starch were attacked by one.

The presence of a few supposedly non-liquefiers among the motile strains may as probably—and even more probably—be an indication of the inaccuracy and unreliability of the gelatin liquefaction test than of the presence of true intermediate organisms, for the number of gelatin liquefiers recognized increases with the period of incubation. Again is it not reasonable to explain the presence of several glycerol and starch fermenters among the motile strains as due to mixed cultures? Picking off a colony from a plate, even after several replatings, is no absolute criterion that a pure culture was obtained. Some species stick tenaciously together.

One of the motile starch fermenting strains referred to above

was plated out on brilliant green agar. Ten colonies were fished into motility agar and starch; three were non-motile starch fermenters, three were motile and did not attack starch, while four were both motile and starch fermenters thus indicating that, in this instance at least, the presumably overlapping or intermediate

TABLE 2

Showing correlation of indol, dulcitol, and inulin for 62 strains of B. aerogenes

		Indol +	Indol −	Dulcitol +	Dulcitol −	Inulin* +	Inulin* −
Indol	+	31		20	11	7	18
	−		31	14	17	11	18
Dulcitol	+	20	14	34		8	20
	−	11	17		28	10	16
Inulin*	+	7	11	8	10	18	
	−	18	18	20	16		36

* Eight cultures not tested in inulin.

TABLE 2A

Coefficient of correlation for each pair of characters in table 2

	Indol	Dulcitol	Inulin
Indol		+.39	-.22
Dulcitol	+.39		-.22
Inulin	-.22	-.22	

strains are in all probability merely mixed cultures. It is not contended that intermediate strains do not occur; they undoubtedly do; but it is desired to point out that these have been over emphasized in the past and that the plating method cannot always be relied upon to yield pure cultures.

B. cloacae may be defined as a gram negative short rod which ferments lactose weakly; forms acetylmethylcarbinol from glucose; is alkaline to methyl-red; motile; rarely forms indol; prac-

tically always forms gas from sucrose, raffinose, mannitol and salicin; and occasionally from dextrin; gelatin is typically liquefied; and glycerol, inulin and starch are not fermented. As noted above, the few glycerol, inulin and starch fermenters are probably due to mixed cultures and may be dismissed.

The three sucrose negative cultures (also raffinose negative) may be regarded as a variety corresponding to the *B. levans* which MacConkey records as very rare.

The dextrin fermenters probably also constitute a variety of *B. cloacae* but as the composition of dextrin is so variable we hesitate to employ it for differential purposes for the present.

B. aerogenes resembles *B. cloacae* in several respects. It forms acetylmethylcarbinol from glucose; is alkaline to methyl-red; and ferments sucrose, raffinose, mannitol, and salicin with gas formation. On the other hand lactose is more vigorously attacked; gelatin is typically not liquefied; the organisms are non-motile; while glycerol and starch are fermented with gas formation.

Indol was formed by 31 (50 per cent); gas from dulcitol was formed by 31 (50 per cent); and from inulin by 18 ($33\frac{1}{3}$ per cent) of the *B. aerogenes* strains (8 cultures were not tested with inulin). From tables 2 and 2A which show the distribution with respect to indol, inulin and dulcitol, and the correlation coefficients for these reactions, it is evident that the characters are not associated. They may be of significance for separation of varieties. The utter lack of correlation necessitates the employment of all of these characters, which would lead to the formation of eight varieties. It is deemed unwise to establish such varieties until more extensive collections are studied.

THE COLI GROUP

In the *B. coli* group are included 182 strains quite evenly distributed between the different animal sources, sewage and soil. The group differs sharply from the *B. aerogenes-B. cloacae* series in that the Voges-Proskauer reaction is negative and the methyl-red reaction positive. Starch is not attacked by any of the strains. It has been shown by the author that the Voges-Pros-

kauer negative strains attack the monosaccharids more vigorously, but the disaccharids, trisaccharid, and glucoside no less vigorously than the Voges-Proskauer positive strains.

In table 3 is shown the correlation between the various reactions. As all strains attacked galactose, lactose, maltose and

TABLE 3

Showing correlation of characters among 182 strains of the coli group

		Motility		Indol		Sucrose		Raffinose		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
Motility	+	130		114	16	77	53	77	53	80	50	92	37	89	41
	−		52	49	3	16	36	19	33	17	35	33	19	25	27
Indol	+	114	49	163		84	79	86	77	87	76	110	52	109	54
	−	16	3		19	9	10	10	9	10	9	15	4	5	14
Sucrose	+	77	16	84	9	93		89	4	64	29	56	36	63	30
	−	53	36	79	10		89	7	82	33	56	69	20	51	38
Raffinose	+	77	19	86	10	89	7	96		65	31	60	35	66	30
	−	53	33	77	9	4	82		86	32	54	65	21	48	38
Dulcitol	+	80	17	87	10	64	33	65	32	97		63	34	75	22
	−	50	35	76	9	29	56	31	54		85	62	22	39	46
Glycerol	+	92	33	110	15	56	69	60	65	63	62	125		89	36
	−	37	19	52	4	36	20	35	21	34	22		56	25	32
Salicin	+	89	25	109	5	63	51	66	48	75	39	89	25	114	
	−	41	27	54	14	30	38	30	38	22	46	36	32		68

mannitol with gas formation and failed to attack the polysaccharids, dextrin, inulin and starch, or to liquefy gelatin, these substances are not included in the table.

The proportion of positive reactions for all strains of the *B. coli* group is as follows: motility 130 (71.5 per cent); indol 163 (89.6 per cent); sucrose 93 (51.1 per cent); raffinose 96 (52.7 per cent); dulcitol 97 (53.3 per cent); glycerol 125 (68.8 per cent) and salicin 114 (62.7 per cent).

The correlation coefficients for each pair of characters is given in table 3A.

The highest coefficient obtained for motility is 0.53 with both

sucrose and dulcitol. Its correlation with other characters is therefore not very marked.

Indol seems to be correlated with salicin, but the small proportion of indol negative strains (10.4 per cent) makes the association of questionable value, and as the coefficients with other substances are extremely low, indol may be eliminated.

Glycerol correlates somewhat with salicin (coefficient 0.52) but

TABLE 3A

Coefficients of correlation for each pair of characters in table 3

	Motility	Indol	Sucrose	Raffinose	Dulcitol	Glycerol	Salicin
Motility		-.39	+.53	+.43	+.53	+.18	+.40
Indol	-.39		+.08	+.00	+.02	-.28	+.76
Sucrose	+.53	+.08		+.99	+.58	-.38	+.20
Raffinose	+.43	+.00	+.99		+.58	-.29	+.27
Dulcitol	+.53	+.02	+.58	+.58		-.21	+.60
Glycerol	+.18	-.28	-.38	-.29	-.21		+.52
Salicin	+.40	+.76	+.20	+.27	+.60	+.52	

with no other character and is therefore not considered desirable for subdivision at this point.

The choice of a differential character is thus narrowed down to sucrose, raffinose, dulcitol and salicin. Sucrose and raffinose are almost perfectly associated (coefficient of correlation 0.99). Consideration of either therefore suffices for both and as the former is slightly better correlated with other characters, sucrose is selected for further discussion.

A comparison of salicin with dulcitol indicates that the alcohol is to be preferred. Salicin correlates better with glycerol and indol (the latter relation of questionable value), while dulcitol has higher coefficients with motility, sucrose and raffinose. A similar consideration leads to the choice of sucrose over salicin.

Sucrose and dulcitol therefore remain. These are the two characters in regard to which there is considerable difference of opinion among students of the *B. coli* group. MacConkey gives preference to sucrose, and in this selection is supported by many investigators (Howe 1912, Kligler 1915, Rogers 1915, etc.); while Jackson (1911) and more recently Giltner (1916) subdivide first on dulcitol. It is quite interesting, therefore, that on the basis of the correlation coefficients there is really little choice between the two. Both are equally well correlated with motility (coefficient 0.53); partially with each other (coefficient 0.58) and not associated with indol. Dulcitol correlates partially with salicin (coefficient 0.60), while sucrose does not (coefficient 0.20). On the other hand, sucrose is almost perfectly correlated with raffinose (coefficient 0.99), whereas salicin is only partially (coefficient 0.58). Although neither can be regarded as associated with glycerol, the coefficient with sucrose (−0.38) is greater than with dulcitol (−0.21).

If our selection is to be guided entirely by correlation, the choice between dulcitol and sucrose is a toss up. Sucrose was finally selected for the primary division because it is more widely distributed in nature, more available to students for investigational purposes, more widely accepted by bacteriologists, and its fermentation better correlated with the source than is dulcitol decomposition. Differentiation on sucrose yields a sucrose positive group of 93 strains, and a sucrose negative group of 89 strains.

THE SUCROSE NEGATIVE STRAINS OF THE COLI GROUP

Of the 89 strains which did not form gas from sucrose, 33 (37.1 per cent) were positive in dulcitol; 69 (77.6 per cent) positive in glycerol; and 51 (57.3 per cent) gave gas in salicin; 53 (59.6 per cent) were motile; only 10 (11.3 per cent) failed to form indol.

The distribution of the organisms, with regard to motility, dulcitol, glycerol, salicin and indol is given in table 4, and the coefficient of correlation for each pair of reactions is given in table 4A. For these strains motility is not distinctly correlated with

any other character. Dulcitol and glycerol are not correlated with each other, nor with indol and motility, but each has a high coefficient of association with salicin. The coefficient for dulcitol

TABLE 4

Showing correlation of characters among 89 sucrose negative strains of the coli group

		Motility		Indol		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−
Motility	+	53		45	8	22	31	43	10	33	20
	−		36	34	2	11	25	26	10	18	18
Indol	+	45	34	79		29	50	61	18	51	28
	−	8	2		10	4	6	8	2		10
Dulcitol	+	22	11	29	4	33		27	6	28	5
	−	31	25	50	6		56	42	14	23	33
Glycerol	+	43	26	61	8	27	42	69		48	21
	−	10	10	18	2	6	14		20	3	17
Salicin	+	33	18	51		28	23	48	3	51	
	−	20	18	28	10	5	33	21	17		38

TABLE 4A

Coefficients of correlation for each pair of characters in table 4

	Motility	Indol	Dulcitol	Glycerol	Salicin
Motility		−.50	+.22	+.25	+.25
Indol	−.50		−.07	−.08	+1.00
Dulcitol	+.22	+.07		+.20	+.78
Glycerol	+.25	−.08	+.20		+.86
Salicin	+.25	+1.00	+.78	+.86	

with salicin is 0.78 and for glycerol with salicin is 0.86. Indol is also correlated with salicin; all of the 10 indol negative strains are also salicin negative. Differentiation is therefore made upon salicin which gives a sucrose negative, salicin positive subgroup

of 51 strains, and a sucrose negative, salicin negative subgroup of 38 organisms.

The sucrose-negative, salicin-positive subgroup (*B. coli*). The distribution of the 51 sucrose negative salicin positive strains on motility, dulcitol, and glycerol is indicated in table 5. 33 (64.9 per cent) are motile; 28 (54.9 per cent) form gas from dulcitol, and 48 (94.3 per cent) from glycerol. The extremely small proportion of glycerol negative strains (5.7 per cent) eliminates

TABLE 5

Showing correlation of characters among 51 sucrose negative—salicin positive strains of the coli group. (*B. coli*)

		Motility		Dulcitol		Glycerol	
		+	−	+	−	+	−
Motility	+	33		18	15	30	3
	−		18	10	8	18	
Dulcitol	+	18	10	28		25	3*
	−	15	8		23	23	
Glycerol	+	30	18	25	23	48	
	−	3		3			3

* Coefficient of correlation for motility and dulcitol = 0.02.

this alcohol from further statistical consideration. From table 5 it is seen that motility and dulcitol are not correlated. Further subdivision on correlated characters is not feasible. This entire group then is regarded as the species *B. coli* and two varieties may be formed on motility—the motile *B. coli-communis* and the non-motile *B. coli-immobilis*.

The sucrose negative, salicin negative subgroup (*B. acidi-lactici*). Of the 38 organisms which were negative for both sucrose and salicin, 20 (52.7 per cent) were motile, 28 (73.7 per cent) formed indol, 21 (55.3 per cent) were positive with glycerol, while only 5 (13.2 per cent) formed gas from dulcitol as shown in table 6. From table 6A it appears that motility is correlated with dulcitol fermentation and indol, and has a slightly higher coefficient with

glycerol than has dulcitol. Indol has a slightly higher coefficient with dulcitol than motility, but the number of dulcitol positive strains is so small, that the coefficients observed cannot be relied upon. Indol and motility seem to be correlated (coefficient

TABLE 6

Showing correlation of characters among 38 sucrose negative, salicin negative strains of the coli group. (B. acidi-lactici)

		Indol		Motility		Dulcitol		Glycerol	
		+	−	+	−	+	−	+	−
Indol	+	28		12	16	2	26	14	14
	−		10	8	2	3	7	7	3
Motility	+	12	8	20		4	16	13	7
	−	16	2		18	1	17	8	10
Dulcitol	+	2	3	4	1	5		2	3
	−	26	7	16	17		33	19	14
Glycerol	+	14	7	13	8	2	19	21	
	−	14	3	7	10	3	14		17

TABLE 6A

Coefficients of correlation for each pair of characters in table 6

	Indol	Motility	Dulcitol	Glycerol
Indol		+.68	+.69	+.40
Motility	+.68		+.62	+.40
Dulcitol	+.69	+.62		+.34
Glycerol	+.40	+.40	+.34	

0.68), and their coefficients with glycerol are identical (0.40). In a preliminary report subdivision was made upon motility into the motile species *B. Gruenthal*, and non-motile *B. acidi-lactici*. It seems best, until more extensive collections are studied, that

all of the sucrose-negative, salicin-negative strains be included in the species *B. acidi-lactici* in which may be recognized two varieties, the motile *B. acidi-lactici* var. *Gruenthali* and the non-motile *B. acidi-lactici* var. *immobili*.

TABLE 7

Showing correlation of characters among 93 sucrose positive strains in the coli group

		Motility		Indol		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−
Motility	+	77		69	8	58	19	49	27	56	21
	−		16	15	1	6	10	7	9	7	9
Indol	+	69	15	84		58	26	49	34	58	26
	−	8	1		9	6	3	7	2	5	4
Dulcitol	+	58	6	58	6	64		36	28	47	17
	−	19	10	26	3		29	20	8	16	13
Glycerol	+	49	7	49	7	36	20	56		41	15
	−	27	9	34	2	28	8		36	21	15
Salicin	+	56	7	58	5	47	16	41	21	63	
	−	21	9	26	4	17	13	15	15		30

TABLE 7A

Coefficients of correlation for each pair of characters in table 7

	Motility	Indol	Dulcitol	Glycerol	Salicin
Motility		−.27	+.67	+.40	+.54
Indol	−.27		+.05	−.42	+.28
Dulcitol	+.67	+.05		−.32	+.39
Glycerol	+.40	−.42	−.32		+.32
Salicin	+.54	+.28	+.39	+.32	

THE SUCROSE POSITIVE STRAINS OF THE COLI GROUP

Of the 93 organisms which fermented sucrose with gas formation, 77 (82.8 per cent) were motile; 84 (90.4 per cent) formed indol; and positive gas reactions were obtained as follows: dulcitol 64 (72.1 per cent); glycerol 56 (60.2 per cent) and salicin

63 (67.8 per cent). The distribution with respect to these characters and the correlation coefficients are shown in tables 7 and 7A respectively, where it is seen that motility correlates better with dulcitol than does any other of the characters. It also correlates best with salicin. Motility is the best correlated

TABLE 8

Showing correlation of characters among 77 sucrose positive motile strains of the coli group. (B. communior)

		Indol		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−
Indol	+	69		53	16	43	25	51	18
	−		8	5	3	6	2	5	3
Dulcitol	+	53	5	58		32	26	41	17
	−	16	3		19	17	1	15	4
Glycerol	+	43	6	32	17	49		37	13
	−	25	2	26	1		27	19	8
Salicin	+	51	5	41	15	37	19	56	
	−	18	3	17	4	13	8		21

TABLE 8A

Coefficients of correlation for each pair of characters in table 8

	Indol	Dulcitol	Glycerol	Salicin
Indol		+.33	−.27	+.26
Dulcitol	+.33		−.87	−.22
Glycerol	−.27	−.87		+.09
Salicin	+.26	−.22	+.09	

character. There are thus two subgroups, a sucrose-positive, motile subgroup of 77 strains and a sucrose positive non-motile subgroup comprising 16 strains.

The sucrose positive motile subgroup (*B. communior*). Inspection of tables 8 and 8A shows that among the sucrose positive motile strains, neither indol production nor salicin fermentation is correlated with other characters. Gas formation from dulcitol

and glycerol shows a strong negative association. Those strains which failed to attack glycerol practically always fermented dulcitol. Thus 26 of 27 glycerol negative are dulcitol positive while 17 of 18 dulcitol non-fermenters, tested, formed gas from glycerol. To put it another way, inability to attack either of the alcohols is accompanied by fermentation of the other. Fermentation of either, however, yields but little information as to

TABLE 9

Showing correlation of characters among 16 sucrose positive, non-motile strains, of the coli group

		Dulcitol +	Dulcitol −	Glycerol +	Glycerol −	Salicin +	Salicin −
Dulcitol	+	6		4	2	6	
	−		10	3	7	1	9
Glycerol	+	4	3	7		5	2
	−	2	7		9	2	7
Salicin	+	6	1	5	2	7	
	−		9	2	7		9

TABLE 9A

Coefficients of correlation for each pair of characters in table 9

	Dulcitol	Glycerol	Salicin
Dulcitol		+.65	+1.00
Glycerol	+.65		+.80
Salicin	+1.00	+.80	

how the other will react. The desirability of subdividing on either glycerol or dulcitol to form two species is questioned. For the present the entire group of sucrose fermenting motile forms is designated as *B. communior* and two varieties may be formed on glycerol or dulcitol.

The sucrose-positive non-motile subgroup. Only 16 of the sucrose fermenters were non-motile, and only one of these failed to produce indol. Although the number of organisms is small, it is quite surprising that they should be so evenly divided with respect to gas formation from the test substances. Thus 6

(37.5 per cent) are positive with dulcitol, 7 (43.7 per cent) with glycerol, and 7 (43.7 per cent) with salicin. From tables 9 and 9A it is seen that salicin is the best correlated character. Of the seven salicin positive strains, 6 (85.8 per cent) attack dulcitol and 5 (71.5 per cent) glycerol. The characteristics of this group

TABLE 10

Distribution of organisms from different sources among the various species and varieties

		B. cloacae	B. aerogenes	B. communior	B. neapolitanus	B. coscoroba	B. coli		B. acidi-lactici		Total
							communis	immobilis	Gruenthali	immobili	
Soil	No	88	54	26	0	0	2	0	7	0	177
	%	49.7	30.5	14.7			1.1		4.0		
Horse	No	0	0	15	0	0	4	0	0	0	19
	%			79.0			21.0				
Sheep	No	0	0	16	0	5	1	0	0	0	22
	%			72.8		22.7	4.5				
Cow	No	0	0	6	4	0	9	0	1	0	20
	%			30.0	20.0		45.0		5.0		
Pig	No	0	0	9	0	1	11	1	9	0	31
	%			29.0		3.2	35.6	3.2	29.0		
Sewage	No	1	8	3	3	2	1	12	2	7	39
	%	2.6	20.5	7.7	7.7	5.1	2.6	30.8	5.1	17.9	
Man	No	0	0	2	0	1	5	5	1	11	25
	%			8.0		4.0	20.0	20.0	4.0	44.0	
Total		89	62	77	7	9	33	18	20	18	333

therefore resemble the *B. neapolitanus* of MacConkey's varieties. On the other hand, 7 (77.8 per cent) of the 9 salicin negative strains are negative for glycerol while all failed to ferment dulcitol. These are therefore the *B. coscoroba* of MacConkey's classification.

RELATION OF SPECIES TO SOURCE

Table 10 shows the distribution of the organisms from different sources among the various species and varieties. Species or varieties and habitat seem to be somewhat related.

B. aerogenes and *B. cloacae* were obtained only from soil and sewage and were not isolated from any of the animals tested. *B. cloacae* constituted 49.7 per cent of the soil and 2.6 per cent

of the sewage strains, while 30.5 per cent from soil and 20.5 per cent from sewage were *B. aerogenes*.

B. communior was isolated from all sources as follows: soil 14.7 per cent; horse 79 per cent; sheep 72.8 per cent; cow 30 per cent; pig 29 per cent; sewage 7.7 per cent and man 8 per cent. The relative abundance of *B. communior* among the lower animals and scarcity in man and sewage, may well be investigated further.

B. neapolitanus was present only in bovine feces and sewage, comprising 20 per cent of the bovine and 7.7 per cent of the sewage strains.

Of the 9 *B. coscoroba*, 5 were from sheep, 1 from pig, 2 from sewage, and 1 from man. 22.7 per cent of sheep, 3.2 per cent of pig, 5.1 per cent of sewage and 4 per cent of human strains fall in this species.

B. coli, like *B. communior* was isolated from all of the sources tested, but a rather distinct correlation with the source is observed with the varieties *B. coli-communis* and *B. coli-immobilis*. The former comprise 1.1 per cent of soil; 21 per cent of horse; 4.5 per cent of sheep; 45 per cent of cow; 35.6 per cent of pig; 2.6 per cent of sewage; and 20 per cent of human strains. *B. coli-immobilis* was not obtained from the soil, horse, sheep or cow, but it made up 3.2 per cent of the pig, 30.8 per cent of the sewage, and 20 per cent of the human strains.

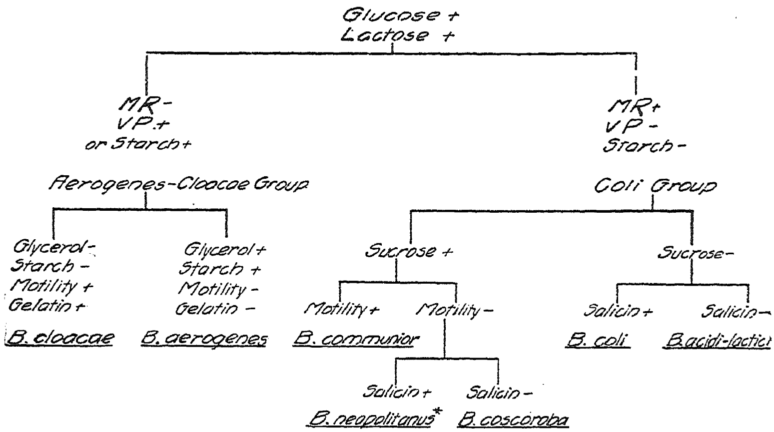
B. acidi-lactici was not obtained from the horse nor sheep, and only rarely from the cow (5. per cent) or soil (4 per cent). The motile variety *B. acidi-lactici var. Gruenthali* was particularly abundant among the pig cultures (29 per cent) and rare in sewage (5.1 per cent) and man (4 per cent). The non-motile *B. acidi-lactici var. immobili* was restricted to man and sewage entirely, comprising 44 per cent of the human and 17.9 per cent of the sewage strains.

If subsequent and more extensive studies confirm these results the determination of species and varieties would have some bearing on the interpretation of the colon test.

The author takes this opportunity to express his gratitude to Dr. R. E. Buchanan for many helpful suggestions and encouragement, and to Prof. G. W. Snedecor for assistance and elucidation of the mathematical principles involved.

SUMMARY

From a statistical study of 333 coli-like bacteria isolated from soil, sewage, and the feces of various animals, the following classification is suggested:



* Designation as species questionable. Probably preferable to regard it as a variety of *B. communior*.

TABLE 11

Per cent of positive reactions

	V.P.	Indol	Gelatin	Motility	Starch	Inulin	Dextrin	Salicin	Raffinose	Sucrose	Dulcitol	Glycerol	No. of Strains
B. cloacae	100.0	16.8	91.0	100.0	4.5	4.5	30.4	98.0	93.3	96.7	15.7	9.0	89
B. aerogenes	100.0*	50.0	3.2	0.0	98.5	29.1	100.0	100.0	100.0	100.0	50.0	98.5	62
B. communior	0.0	89.6	0.0	100.0	0.0	0.0	0.0	72.8	94.8	100.0	75.4	63.7	77
B. neapolitanus	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	100.0	85.8	71.5	7
B. coscoroba	0.0	89.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0	22.2	9
B. coli	0.0	100.0	0.0	64.7	0.0	0.0	0.0	100.0	11.8	0.0	55.2	94.3	51
B. acidi lactici	0.0	64.0	0.0	52.7	0.0	0.0	0.0	0.0	2.6	0.0	13.2	55.2	38

* Ten questionable reactions included.

The per cent of positive reactions of the different species is indicated in table 11. *B. neapolitanus* differs from *B. communior*

only with respect to motility, and it may therefore be well to regard it as a variety of *B. communior*. However *B. coscoroba* is so distinctly different from the other sucrose fermenters that its designation as a species seems justified. It should be borne in mind however, that the differentiation in this instance is based on only 9 individual cultures so that the correlations observed must not be over emphasized.

CONCLUSIONS

To treat all characters as of equal taxonomic significance leads to an infinite number of unstable varieties; a condition to be avoided.

Subdivision on correlated characters results in a small number of groups which possess considerable stability.

The species described are quite strikingly correlated with the source, and, if more extensive investigations confirm these observations, recognition of the various species may be of sanitary significance.

It is not supposed that the classification presented is the last word in the differentiation of coli-like bacteria, but it is hoped that if subdivision is to be made upon correlated characters—and there is much to commend such a procedure—the method described in this paper for the determination of the best correlated character, by a study of the coefficients of correlation, will be an aid to later investigators.

REFERENCES

Bergey and Deehan, S. J. 1908 J. Med. Research, **19**, 175.
Durham, H. E. 1901 J. Exper., **5**, 353.
Ford, W. W. 1903 Studies from the Rockefeller Inst. of Med. Res. 11.
Howe, E. C. 1912 Science, N. S., **35**, 225.
Jackson, D. D. 1911 Am. J. Pub. Health, **1**, 930.
Johnson and Levine 1917 J. Bact.
Kligler, I. J., 1914 J. Infect. Dis., **15**, 135.
Levine, M. 1916 J. Infect. Dis., **19**, 773.
MacConkey, A. 1905 J. Hyg. **5**, 333; 1909 J. Hyg. **9**.
Rogers, et al.. J. Infect. Dis., 1914, **14**, 411; 1914, **15**, 100; 1915, **17**, 137.
Smith, Th. 1893 The Wilder Quarter Century Book. 187.
Yule, 1916 An introduction to the theory of statistics.

STUDIES ON FOWL CHOLERA

V. THE TOXINS OF BACILLUS AVISEPTICUS[1]

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In presenting the results of his very suggestive studies on the agglutination of bacteria in vivo, Dr. Bull (1916) makes the following statement:

> From table 1 it is seen that bouillon cultures of *Bacillus avisepticus* are highly toxic for rabbits, 0.5 cc. of culture per kilo causing acute death. The intoxication is largely due to a toxin, since 1 cc. per kilo of body weight of a bacteria-free filtrate from a twenty-four hour culture causes acute death.

and again:

> These results especially emphasize the significance of agglutinins and opsonins in the mechanism of natural resistance to infection, since *Bacillus avisepticus* produces a powerful toxin and is still incapable of causing a septicemia of any consequence in the presence of these antibodies.

In view of the difficulty of reconciling these conclusions with studies conducted by other investigators upon *Bacillus avisepticus*, as well as with observations made by the present writer (Hadley 1912, 1914a, 1914b, 1917) in studies on infection and resistance in fowl cholera, it seems desirable to bring into relief the somewhat extraordinary nature of the statements made by Dr. Bull with reference to toxin-production by virulent cultures of the fowl cholera bacteria. This is perhaps especially desirable at this time in view of the many current misconceptions regarding

[1] Contribution 233 from the Agricultural Experiment Station of the Rhode Island State College.

the nature and biological features of the bacterial species in question

In a series of classic papers which appear to have attracted less attention than they deserve among American students of immunological problems, Edmund Weil (1905a, 1905b, 1907a, 1907b, 1908) following the steps of Bail, has presented the clearest evidence as to the elements favoring infection which characterize the activity of the bacteria of the fowl cholera group. An analysis of the situation as based upon his studies and those of his collaborators (Weil and Braun, 1909) may briefly be summarized as follows:

1. Rabbits, inoculated in the pleural cavity with virulent fowl cholera cultures produce an exudate which harbors substances favoring infection—the *aggressins.*

2. These aggressins, which are manifestly equivalent to the virulins of Rosenow and to the antiphagines of Tchistovitch and Yourévitch, possess in high degree the property of furthering infection. Treated animals die with a sub-lethal dose of virus while control animals resist ten times that dose.

3. The infection-furthering property of the exudate does not lie in poisonous substances, since animals easily tolerate 12 cc. or eight times the dose necessary to determine a fatal issue when administered together with a non-lethal dose of virus.

4. The results cannot be due to an inactivating influence in the opsonic sense, since leukocytes (whether in immune or normal animals) manifest no, or slight, phagocytosis either in vitro or in vivo.

5. Toxicity and aggressivity are not identical; toxicity is most noteworthy in organisms which are not highly aggressive; and the most aggressive organisms are non-toxic. *B. avisepticus*, the causative agent in cholera of fowls, is the most aggressive organism that has been studied.

Weil's tests, devised to show whether toxins can be held responsible for the highly fatal results following inoculation with fowl cholera virus, are elaborately planned and (so far as one may judge) carefully executed. Pasteur's observation on the toxicity of the concentrated filtrates of *B. avisepticus* has never been

confirmed under conditions in which it was clearly shown that no bacteria were present in the filtrate. Stang, moreover, was able to inject 20 cc. of concentrated filtrate without producing an infection, or signs of poisoning.

Weil concludes that one cannot demonstrate the slightest toxicity against the most susceptible animals (like the rabbit) in the case of fowl cholera. He pertinently inquires why a toxin-neutralization if present in immune animals should diminish the unlimited development of the bacteria. *B. avisepticus* shows characters sharply opposed to those of toxin-forming bacteria which never develop in great numbers in the body (diphtheria, tetanus, dysentery).

Another line of investigation which demonstrates the lack of extracellular toxins in the case of *B. avisepticus* is found in the experiment of Citron and Pütz (1907) who employed serum extracts and water-extracts of virulent bacterial cultures (artificial aggressins as opposed to natural aggressins of Weil). With these artificial aggressins the authors mentioned inoculated rabbits with the aim of furthering infection, or of producing immunity. The method did not in either case prove so successful as the method of Weil, involving pleural exudates, but the point for our present consideration is that the experiments conducted were such as to demonstrate the total lack of either exotoxins or endotoxins in the materials used for injections. The inoculated animals invariably remained in good health up to the time of infection with the active virus.

Other evidence showing the absence of toxins in the case of *B. avisepticus* is supplied indirectly by the studies of Weil and Braun (1909). Weil (1905b) had already shown that his method of inoculation with sterile pleural exudates was not only sufficient to protect animals against infection, but that the serum of such animals possessed protective power. The question naturally arose regarding the nature of the protective substances in such serum. Weil and Braun attempted to ascertain whether the protection was due to bactericidal substances. Animals were accordingly inoculated with serum which had been "treated" (absorbed) with fowl cholera bacteria, and survived infection.

When the treated serum was tested for its bactericidal properties it was found that they had quite disappeared. Untreated serum still showed a slight growth-inhibiting power. The authors were therefore led to conclude that bactericidal antibodies cannot be considered in the protective action since they can be separated from such serum without causing the degree of protection to be diminished. In a similar manner Weil was able to show later that old immune serum might retain its bactericidal properties after it had lost all protective power.

The same authors demonstrated by detailed experiments that there is no opsonic action in fowl cholera immune serum. It was clearly shown that there was no phagocytosis of the fowl cholera bacteria in either normal or immunized animals. Sulima (1909) on the contrary, showed that some phagocytosis took place in immune animals but that it was not marked. It is obvious that in dealing with such small organisms as the bacteria of fowl cholera (0.3 to 0.5 μ) one must use suitable methods of staining, and much care in examining the leukocytes for evidences of phagocytosis.

In the work of Huntemüller (1906) also there is good evidence of the lack of toxin-production by *B. avisepticus*. One phase of this writer's investigations involved the injection into rabbits of large amounts of bacterial filtrates in order to ascertain whether protective substances could be washed out of the cells. This was not found to be the case; but the point of present interest lies in the fact that the injection of the filtrates produced no ill effect as they certainly would have done if the organisms used had been capable of elaborating so powerful an exotoxin as Dr. Bull's statements imply.

Taken as a whole, the results of studies on infection and immunity in fowl cholera, although appearing to demonstrate that the protective function of fowl cholera immune serum does not rest upon the action of opsonins, nor upon bacteriolytic or bactericidal components (thus, by elimination, suggesting an antitoxic immunity), have failed to reveal the activity of any agent that can properly be regarded as a toxin, either intra- or extra-cellular.

Within the past few years the present writer (Hadley and

Amison, 1911, Hadley, 1912, 1914a, 1914b) has devoted some attention to the problems of infection and resistance in the organisms of the hemorrhagic septicemia group. Use has been made of a highly virulent culture (strain 48) of *B. avisepticus.* The lethal dose of this culture for rabbits has frequently stood at 0.000,000,000,001 cc. of a forty-eight-hour broth culture. Gallagher (1917) in recent experiments, in which he employed the same culture as the infective agent to test the immunizing power of the writer's Culture 52, as well as of certain strains of cattle, sheep and swine septicemia, used the strain in amounts of 0.000,000,001 cc. with regularly successful infections. Such a strain should be well adapted for testing out the question of toxin-production by *B. avisepticus.*

EXPERIMENTAL

In studies involving the attempt to immunize rabbits and pigeons with killed cultures of strain 48, not yet reported, the writer has had occasion to administer large doses of killed broth culture. The following is a protocol:

Eight rabbits (923–931) each received subcutaneously on October 17, 1912, 1 cc. of a forty-eight hour broth culture of strain 48 killed by heating at 63°C. for thirty minutes; no illness resulted. Dose repeated on November 21 and again on December 5; no result.

On December 20, 1912, all the animals, together with two controls, were infected by inoculation with 0.01 cc. of forty-eight hour broth culture of strain 48. The controls and all but two of the principals died in fourteen to forty hours.

From the above test it may be concluded that the cultures killed at a temperature usually regarded as too low to destroy in toto extracellular toxins, if present, showed no toxicity for rabbits in the amounts used; furthermore that they failed in all but two instances to afford protection against infection with living culture.

Tests were also performed on pigeons, which are highly susceptible to intramuscular inoculation with the fowl cholera virus.

Six pigeons were inoculated subcutaneously, at intervals of

seven to ten days with forty-eight-hour broth cultures of strain 48, killed by heating at 63° for one-half hour.[2]

PROTECTIVE INOCULATIONS							INFECTION WITH CULTURE 48. MARCH 17		
INOCULATION RECORD	Dates and amounts					Method	Method	Result	
	2-3	2-10	2-17	2-24	3-3			Date of Death	Period
1240	1.0	2.0	2.0	2.0	2.0	S	S	March 23	Six days
1241	1.0	2.0	2.0	2.0	2.0	M	S	March 28	Eleven days
1242	1.0	2.0	2.0	2.0		S	S	March 18	One day
1243*	1.0	2.0	2.0	2.0		M	S	April 4*	Survived
1244	1.0	2.0				S	S	March 18	One day
1245	1.0	2.0				M	S	March 23	Six days
1276							S	March 18	One day
1277							M	March 18	One day

S, subcutaneous (in the neck).
M, intramuscular (in the breast muscle).
* Reinforced with 0.000,01 cc. culture 48, March 30.

No reactions followed the immunizing inoculations with killed cultures. Two weeks after the last immunizing dose all the principals together with two controls were infected with 0.000,01 cc. of culture 48, as shown in the table, and with the results indicated.[3]

These protocols are similar to many others on record and indicate how free the *B. avisepticus* cultures were from anything that could be regarded as bacterial toxins. One can readily imagine the results that would follow had *B. diphtheriae* or some other toxic culture been used in these experiments in place of the fowl cholera bacterium

The instances mentioned above involved the use of killed cultures and the doses were not administered intravenously as was the case in Dr. Bull's tests on rabbits and dogs in which he obtained results that suggested acute intoxication. In order

[2] In this particular test, in order to obtain a more concentrated suspension of bacteria, the cultures were centrifuged and two-thirds of the supernatant medium poured off. The sediment was re-suspended in the remaining one-third and injected.

[3] These data will be presented at a later date in connection with the subject of immunization by means of killed cultures.

to reproduce more faithfully the experimental conditions under which he worked the writer inoculated intravenously two rabbits with 1 cc. per kilo body weight of a forty-eight-hour killed broth culture of the highly virulent strain 48 killed by heating at 62° for 20 minutes. The results are indicated in the accompanying table.

INOCULATION RECORD	WEIGHT	INOCULATION (INTRAVENOUS)			HISTORY OF ANIMAL
		Time	Culture	Amount	
	grams			*cc.*	
1584A	950	May 1, 4 p.m.	Killed at 62°C. for 20 minutes	1.0	5.00 p.m., sick 11.00 p.m., no change. 7.00 a.m., May 2, quiet, does not eat 7.00 a.m., May 3, condition improved
1585A	880	May 1, 4 p.m.	Living	0.9	5.00 p.m., sick 11.00 p.m., very weak, breathing with difficulty 7.00 a.m., dead; general septicemia

From the data presented in the tabulation it is apparent that the intravenous injection of killed culture produced no indications of strong toxicity; moreover, that inoculation with a relatively large amount of living culture produced fatal results only after a period of fourteen hours, indicating no strong toxic action in the unmodified virus.

In addition to the tests mentioned above, another experiment may be cited which demonstrates even more conclusively the absence of a strong extra-cellular toxin derived from a highly virulent culture of *B. avisepticus* growing in broth culture.

A rabbit was inoculated by the intra-abdominal route with 0.001 cc. of a broth culture of strain 48 and died in fourteen hours. The culture was regained from the heart's blood and plated. A colony was subcultured into flasks of chicken broth. One of these was grown at 37°C. for forty-eight hours and passed through a Berkefeld N-candle under suction. The filtrate was tested for sterility as follows: Ten 0.1 cc. samples of filtrate were trans-

ferred to tubes of chicken broth and incubated at 37°C. for ninety-six hours. No growth appeared at the end of that time. In the meantime the remaining filtrate was divided into three portions: portion a was placed in the ice-box; portion b was incubated for twenty-four hours at 37°C., and portion c was heated at 62°C. for twenty minutes. The incubated filtrate showed no growth. It was intended to inject all three portions into rabbits, but, owing to an accident, only the ice-box filtrate and the heated filtrate were employed for rabbits. These were injected intravenously in amounts of 1 cc. as shown in the accompanying table. Of the incubator filtrate 1 cc. was injected intravenously into an adult hen:

INOCULATION RECORD	WEIGHT	FILTRATE	DATE	HISTORY OF ANIMAL
	grams			
1580	2100	Heated	April 5, 4 p.m.	Remains normal
1581	1390	Ice-box	April 5	Remains normal
411H		Incubator	April 5	Remains normal

From these results there is no evidence of a toxic filtrate from the growth of *B. avisepticus*.

The tests described above were made with filtrate from a forty-eight-hour broth culture. Assuming that sufficient time might not have been given for toxin-formation, the second flask was incubated for four days at 37°C., and the culture passed through a Berkefeld N-candle under suction. The fifteen-minute filtrate was tested for sterility by the inoculation of five tubes of chicken broth with 0.1 cc. samples. No growth resulted after forty-eight hours' incubation. The remaining filtrate was divided into two lots. One was placed in the incubator and the other in the ice-box for about twenty-four hours, until used. Intravenous inoculations were made as shown in the following tabulation:

INOCULATION RECORD	WEIGHT	FILTRATE	AMOUNT	TIME	HISTORY OF ANIMAL
	grams		*cc.*		
1581A	1340	Incubated	1.5	April 7	
				11.30 a.m.	Inoculated
				1.45 p.m.	Dull, breathing with difficulty; eyes closed
				3.30 p.m.	No change
				6.30 p.m.	More active
				April 8	
				9.00 a.m.	Quiet, but otherwise normal
1581B	1225	Ice-box	2.0	April 7	
				11.30 a.m.	Inoculated
				1.15 p.m.	Dull, breathing with difficulty; unable to stand
				3.30 p.m.	No change
				6.30 p.m.	Upright, but staggers; breathes with difficulty
				April 8	
				9.00 a.m.	Better; quiet
				April 9	
				9.00 a.m.	Appears normal

In the results of this test it appears that a reaction closely simulating an anaphylactic shock resulted from the inoculation of the second filtrate. The result was not fatal, however, and it may be questioned whether it was due to the presence of an exotoxin or to some other factor at present unrecognized.

In view, however, of the discrepancy between the first and second tests, a third experiment was undertaken in which use was made of a still larger amount of filtrate from a six-day chicken broth culture passed through a Berkefeld N-candle. The filtrate was tested for sterility, and divided into two portions, one of which was incubated over night and the other placed in the ice-box. A slight haziness developed in the incubator tubes and subcultures were made in chicken broth. These, however, remained sterile. A 1314-gram rabbit was accordingly inoculated intravenously with 1.5 cc. of the incubator filtrate. No sign of illness followed within the following twenty-four hours during which the animal was under observation.

As a final test, in order to explain, if possible, the discrepancies between Bull's results and those of the present writer, it seemed desirable to repeat the experiment with the same culture used by Bull. Fortunately, as a result of the careful records kept by the laboratory of the Curator of Public Health, at the American Museum of Natural History, it was possible to locate the culture used by Bull, who had in the meantime discarded his strain, and to obtain a subculture, together with a brief history of organism. This seemed especially desirable in view of the many errors that are made in the recognition of the organism of the authentic fowl cholera. It can scarcely be doubted that the majority of the cultures so labelled, that may be obtained from any laboratory, are not *B. avisepticus*.

Upon receipt of the culture it was plated and subcultured in chicken agar. Flasks of chicken broth were inoculated and incubated for forty-eight hours at 37°C. One of the cultures was then passed through a Berkefeld N-candle under suction and this filtrate tested for sterility. The filtrate was then divided into two portions, one of which was inoculated at 37°C. over night as a final test of sterility; the other kept in the ice-box. The following morning, all evidences having pointed toward the sterility of the filtrate, a 1.5 cc. sample was injected intravenously into a 1375-gram rabbit, which died in less than three hours with symptoms of intoxication.

Another rabbit of 1347 grams was inoculated intravenously with 0.7 cc. of a twenty-four-hour chicken broth culture of Dr. Bull's strain and no ill effects resulted. Apparently 1.5 cc. of a forty-eight-hour culture-filtrate contained a lethal dose of toxin, while 0.7 cc. of a twenty-four-hour culture did not. This is suggestive of an endotoxin rather than an exotoxin. To demonstrate this further, a forty-eight-hour culture of Dr. Bull's strain was shaken for one-half hour, incubated at room temperature for forty-eight hours and shaken again for three hours. It was then passed through a Berkefeld N-candle and the filtrate tested for sterility by subcultures. Of the sterile filtrate 0.5 cc. was then injected into the ear vein of a 1000-gram rabbit. One hour after the

injection the rabbit showed definite symptoms of intoxication and died in about six hours.

The tests reported in the previous pages were sufficient to demonstrate a total lack of toxicity in the culture or filtrates of *B. avisepticus* (strain 48) employed, but to show the presence of a strong toxic substance in the filtrate of forty-eight-hour cultures of the organism used by Dr. Bull. The contrasts may be summarized as follows:

ORGANISM	FILTRATE	CULTURE
B. avisepticus (strain no. 48)	Non-toxic in amounts of 1 cc. per kilo of body-weight	Highly infectious in amounts of (at least) 0.000,000,001 cc. for rabbit of any weight, producing death in fourteen hours
Dr. Bull's culture	Toxic in amounts of 0.5 to 1 cc. per kilo of body weight. Fatal in two to six hours	Small amounts of twenty-four hour culture harmless.*

* May perhaps become lethal after several passages through rabbits.

In view of these discrepancies, it seemed desirable to examine culturally the strain employed by Dr. Bull. It was therefore submitted to the usual cultural and biochemical tests; and in addition was tested for its agglutinative reactions with a number of immune sera, including those of *B. avisepticus* and *Bact. gallinarum* (fowl typhoid). These tests will now be described.

Morphological, cultural and biochemical features of Dr. Bull's strain. Slides prepared from twenty-four-hour chicken agar cultures and stained with gentian violet revealed a short plump rod, having an average size of 0.7 by 0.45μ. a maximum size of 1.2 by 0.5 μ, and a minimum size of 0.4 by 0.3 μ. The ends of the rods were rounded and many of the shorter rods resembled cocci. Pairs of rods were common; chains of three seldom seen; filaments occasionally. Peripheral staining was observed.

The culture grew well on chicken agar; resembled in all respects growth of *Bact. pullorum* or *B. typhosus*. In broth there was a fair clouding at the end of twenty-four hours.

No gas was formed in glucose, sucrose or lactose (Smith tubes). Titration of culture from the open arm of these cultures, and of cultures in other sugar broths (in plain tubes) at the end of five days gave the following results:

CARBOHYDRATE USED IN THE TEST	DR. BULL'S CULTURE	*B. avisepticus* (FOWL CHOLERA)	*Bact. gallinarum* (FOWL TYPHOID)
Glucose	0.8	0.3	1.6
Lactose	0.0		0.0
Sucrose	0.1	0.8	0.0
Dulcitol	0.5	0.9, 0.4, 0.4	0.9, 1.9, 0.9
Dextrin	0.9	0.0, 0.2, 0.2	0.8, 0.8, 0.7
Maltose	1.1	0.1, 0.1, 0.1	0.9, 0.8, 0.8

Litmus milk inoculated with one loopful of a twenty-four-hour broth culture showed a slight acidity in twelve hours at 37°C. This increased slightly for the next twelve to twenty-four hours but after thirty-six to forty-eight hours there was a tendency to return to neutral. The cream ring, however, began to turn blue[4] about twenty-four hours after the inoculation of the tubes and the color steadily increased so to as give a blueness of grade 2[5] before the body of the milk had returned to neutral. Neutralization occurred on the sixth day after inoculation. On the seventh the body of the milk as well as the cream ring was slightly alkaline and on the eighth distinctly alkaline (grade 1). Grade 3 alkaline was attained on the thirtieth day.

Without entering into further details regarding the biochemical reactions, a sufficient number of data have been presented to indicate clearly that the culture with which Dr. Bull worked was not *B. avisepticus* in the sense that this is the causative agent in

[4] It is interesting to note that in alkalining cultures such as *B. pullorum*, *B. gallinarum* and the paratyphoids A and B, the alkalinity may be shown in the cream ring for some days before the color of the body of the milk has even returned to neutral, and many days before the body of the milk becomes alkaline.

[5] See Hadley (1917).

fowl cholera and a member of the hemorrhagic septicemia group. *B. avisepticus* does not ferment most of the carbohydrates ordinarily used in bacteriological work; and it leaves litmus milk quite unchanged after a period of ninety days at 37°C.

Agglutination tests. In view of the fact that fowl cholera immune serum seldom if ever agglutinates completely (in vitro) its homologous antigen in dilutions greater than 1 : 160, the specific agglutination test is obviously of slight value in testing a questionable strain. It is necessary to demonstrate an affinity or lack of affinity with other immune sera. Since the majority of so-called fowl cholera cultures maintained in the laboratories of this country are not *B. avisepticus* but *Bact. gallinarum* (E. Klein), or some other member of the colon-typhoid intermediates, Dr. Bull's culture was first tested against sera immune to the bacillus of fowl typhoid. Since the laboratory collection of immunized animals contained rabbits immune to two fowl typhoid strains obtained from Dr. Theobald Smith, these, among others, were employed for the tests with results as shown in the following table.

From a study of the agglutination features as presented in the table it appears that there was present a satisfactory agglutination affinity between all of the sera immune to the fowl typhoid strains (*Bact. gallinarum*) (115, 116, $102A^6$ and 118^6) and Dr. Bull's culture, but none with *Bact. avisepticus* antigen. Moreover, that serum immune to *B. avisepticus* while agglutinating its homologous antigen at $\frac{C}{40}$, $\frac{4}{80}$, $\frac{2}{160}$, failed to agglutinate Dr. Bull's culture in any significant dilution.

SERUM AND ANTIGEN		SERUM—DILUTIONS								DATE
Culture Number	Designation	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	Control	
115	Fowl typhoid, vs.									
	Homolog. antigen.......	C	C	C	C	C	C	4	T	12-22-16
	B. avisept. antigen......	0	0	0	0	0	0	–	0	1-19-17
	Bull. cult. antigen......	C	C	C	C	C	–	–	0	3-30-17
	Hog cholera antigen.....	T	0	0	0	0	–	–	0	2-16-17
116	Fowl typhoid, vs.									
	Homolog. antigen.......	C	C	C	4	2	T	–	T	4-20-17
	B. avisept. antigen......	0	0	0	0	0	0	–	0	1-20-17
	Bull. cult. antigen......	C	C	4	2	1	–	–	0	4-20-17
	Human typhoid antigen.	1	T	0	0	0	0	–	0	3- 2-17
102A[6]	Fowl typhoid (?), vs.									
	Homolog. antigen.......	C	C	C	C	4	2	T	0	7-31-16
	B. avisept. antigen......	2	1	0	0	0	–	–	0	11- 7-15
	Bull. cult. antigen......	–	C	C	C	C	–	–	0	3-12-17
	Human typhoid antigen.	0	0	0	0	0	–	–	0	3-12-17
118[6]	Fowl typhoid, vs.									
	Homolog. antigen.......	C	C	C	C	C	4	3	0	4-23-17
	B. avisept. antigen......	0	0	0	0	0	–	–	0	4-23-17
	Bull. cult. antigen......	C	C	C	C	C	–	–	0	4-23-17
	Bact. pullorum antigen...	C	C	C	C	C	–	–	0	4-23-17
Mxd.	*Bact. pullorum*, vs.									
	Homolog. antigen.......	C	C	C	C	C	–	–	0	3- 3-17
	B. avisept. antigen......	0	0	0	0	0	–	–	0	1-22-17
	Bull. cult. antigen......	1	T	0	0	0	–	–	0	3-10-17
	Fowl typh. antigen......	C	C	C	C	4	0	–	0	3- 3-17
48, 52	*B. avisepticus*, vs.									
	Homolog. antigen.......	C	4	2	T	0	0	–	T	4-20-17
	Bact. pull. antigen......	0	0	0	0	0	0	–	0	3-26-17
	Bull. cult. antigen......	0	0	0	0	–	–	–	T	4-20-17
	Fowl typh. antigen......	0	0	0	0	–	–	–	0	3-26-17

C = Complete sedimentation.
0 = No agglutination.
4, 3, 2, 1 = Intermediate grade of agglutination.
– = No test.
T = Trace of sediment (control tube.)

[6] Probably *Bact. pullorum* (Note at time of correcting proof).

CONCLUSIONS

These results of the agglutination tests appear to substantiate the results of the cultural tests in demonstrating that Dr. Bull's culture was a strain of the fowl typhoid bacterium and not a representative of the fowl cholera or hemorrhagic septicemia group. The facts that cultures of the fowl cholera bacterium are non-toxic, and that they are not opsonized into phagocytosis, therefore remain uncontroverted so far as Dr. Bull's experiments are concerned. Indeed Tchistovitch (1909) appears to have demonstrated that, in the case of the actual fowl cholera organism, serum from the dog did not assist the rabbit leukocytes to ingest the fowl cholera organisms. Notwithstanding this circumstance, however, Dr. Bull's study of phagocytosis in reference to the culture used by him, if properly interpreted, adds much to our knowledge of the toxicity, opsonins and phagocytosis of one of the most interesting and slightly studied types of the colon-typhoid intermediates.

REFERENCES

Bull 1916 J. Exper. M., **24**, 25–33.
Citron and Pütz 1907 Ztschr. f. Hyg. u. Infektionskrankh., **56**, 145–174.
Gallagher 1917 J. Am. Vet. Ass., **50**, 708.
Hadley and Amison 1911 R. I. Agr. Exper. Sta., Bull. 146.
Hadley 1912 R. I. Agr. Exper. Sta. Bull. 150.
Hadley 1914a R. I. Agr. Exper. Sta. Bull. 157.
Hadley 1914b R. I. Agr. Exper. Sta. Bull. 159.
Hadley 1917 J. Bact. **2**, (73, 263).
Huntemüller 1906 Centralbl. f. Bakteriol., Abt. i, Orig. **42**, 170.
Suliman 1909 Ann. de l'Inst. Pasteur, **23**, 911–920.
Tschistovitch 1909 Ann. de l'Inst. Pasteur, **23**, 834.
Weil 1905a Arch. f. Hyg. **52**, 413–432.
Weil 1905b Arch. f. Hyg. **54**, 149–183.
Weil 1907a Ztschr. f. Hyg. u. Infektionskrankh., **56**, 509–515.
Weil 1907b Arch. f. Hyg., **61**, 293–323.
Weil 1908 Arch. f. Hyg. **65–66**, 81–106.
Weil and Braun 1909 Fol. Serologica. **2**, 151.

A LACTOSE FERMENTING YEAST PRODUCING FOAMY CREAM

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Large amounts of cream are lost during the hot summer months from an undesirable fermentation known as "foamy cream." This loss is due chiefly to the mechanical action of the fermentation, rather than to the effect of undesirable odors and flavors, for often one-third to one-half of the cream is lost from the can through foaming while in transit. The fermentation is best identified by this characteristic foaming action and by the yeasty or fruity odor imparted to the cream.

EXAMINATION OF CREAM

Numerous samples of foamy cream collected from different parts of Kansas by the State Dairy Commissioner have been sent to the bacteriological laboratory for analysis. The microbial flora as revealed by direct microscopic examination and by plate cultures proved to be similar for all samples. The cream was plated on whey and lactose agar respectively. The latter medium was acidified with 1 cc. of a 1 per cent tartaric acid solution per tube of medium.

Yeast cells were very prominent, as well as rod shaped bacteria, which appeared as short or long filaments and showed numerous granules on staining. The latter organism upon isolation proved to be a member of the *B. bulgaricus* group.

All cream showing such a flora caused foaminess when placed in raw or sterile cream; and the inoculated cream always exhibited a microbial flora identical with that of the original product.

The predominating type proved upon isolation to be a lactose fermenting yeast. Its ability to produce foamy cream was easily demonstrated by placing it in raw or sterile cream. The characteristic action of the organism is noted in figure 1.

Fig. 1

PREVIOUS WORK ON LACTOSE FERMENTING YEASTS

In this country, very few investigators have reported the presence of yeasts of this type. An abnormal fermentation in Swiss cheese, as reported by Russell and Hastings (1905) was due to a lactose fermenting yeast. Harrison (1902) observed yeasts capable of fermenting lactose in large numbers in American cheese and milk. He attributed a bitter fermentation in milk and dairy products to their presence.

The majority of lactose fermenting yeasts have been isolated and studied by European investigators. Grotenfelt (1889) described such a yeast isolated from milk and named it *Saccharomyces acidi-lactici*. Beijerinck (1889) isolated a lactose yeast from kefir grains, and one from Edam cheese. The former is known as *Saccharomyces kephyr* and the latter as *Saccharomyces tyrocola*.

While Beijerinck states that both of these yeasts were Saccharomyces, other investigators who have studied them assert that both are incapable of sporulating and hence should be classed as Torulae. A non-spore producing yeast was isolated from Grana cheese by Bochicchio (1894) and named *Lactomyces inflans-caseigrana*. A typical lactose fermenting Saccharomyces obtained from Emmenthaler cheese, is described by Freudenrich and Jensen (1897). Jensen (1902) also noted two species in butter which were true yeasts. Maze (1903) studied ten different torula from soft cheese. One fermented lactose only, while the others fermented glucose, levulose, maltose and sucrose. Duclaux (1900) describes three lactose fermenting yeasts, isolated by Kayser, Adametz and himself. All are non-spore producing yeasts and capable of fermenting glucose, lactose, sucrose, galactose, invert sugar and maltose, slightly. All are apparently different species however. Another typical saccharomyces, *Saccharomyces fragilis*, was isolated from kefir, by Jorgensen (1911).

According to Lafar (1911) the yeasts of Jorgensen, Freudenreich and Jensen, and Jensen and Maze, are the only lactose fermenting yeasts which can definitely be reported as Saccharomyces.

Yeasts capable of fermenting lactose, according to the present classification are grouped as either true or false yeasts. If true yeasts, they sporulate and hence are considered as belonging to Hansen's fifth subgroup of Saccharomyces, if false yeasts, they are non-sporulating varieties, or Torulae. Of the lactose fermenting yeasts studied, representatives of both genera are known. The differentiating factor is sporulation and the observations of different investigators, upon this point, for the same yeasts do not agree. Hansen states that the torula may be only a tempo-

rary stage of development of yeasts. He has demonstrated that, at the least, spore production is not a stable factor, for he has been able to produce an asporogenic race of Saccharomyces by varying the condition of cultivation. This would lead one to doubt the usefulness of attempting to use sporulation in yeasts as of much diagnostic value.

MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL CHARACTERISTICS

The yeast causing "foamy cream" is oval to elliptical in shape, averaging 5 microns in length and 2 microns in breadth.

Typical spores were not demonstrated by cultivation upon gypsum blocks or potato at temperatures of 25°C. and 35°C. According to the present means of classifying yeasts, the organism is therefore a false yeast or torula. The average colony varies from 2 to 3 mm. in diameter; but more minute forms are frequently noted. In appearance the colonies are spherical with smooth edges, having a raised smooth glistening surface. Plate cultures of the organism emit a yeasty or fruity odor. Upon lactose agar it grows moderately, exhibiting a raised, smooth, and dull to glistening growth. It produces a slight cloudiness in sugar broth; no pellicle, acid reaction in litmus milk; and fails to liquefy gelatin. In milk about 0.3 per cent acid is produced. Gas production was demonstrated in glucose, lactose, sucrose, levulose, galactose, maltose and bile lactose; although the action in galactose, maltose and bile lactose was slow and feeble.

The relation of temperature to the rate of fermentation of the organism is represented in table 1. Litmus milk fermentation tubes were inoculated with 0.1 cc. broth culture of the yeast and incubated at 18°C., 25°C. and 37°C., respectively. The results are self explanatory and easily account for the predominance of foamy cream during hot weather. The highest temperature studied, 37°C., is not necessarily the optimum temperature for the development of the organism, but lack of incubation facilities prevented a more exact study of this point.

TABLE 1

The relation of temperature to fermentation

TEMPERATURE OF INCUBATION	DAYS OF INCUBATION						
	1	2	3	4	5	6	7
deg. C.							
18	No change	No change	No change	No change	No change	No change	No change
25	Slight acid	Acid, gas slight	Acid, gas 50 per cent*	Acid, gas 100 per cent			
37	Acid	Acid, gas 100 per cent					

*The percentage of gas refers to the amount formed in the closed arm of a Smith fermentation tube.

POWERS OF RESISTANCE

Thermal death point

Twenty-four hour lactose broth cultures were thoroughly shaken and equal portions placed in sterile capillary pipettes of uniform capacity and thickness of glass. Both ends were sealed by heat and the sealed tubes were then placed in water baths of constant temperatures for ten minutes. At the end of ten minutes the tubes were removed from the water bath and cooled by placing in cold water. The pipettes were opened aseptically and tubes of litmus milk were inoculated with the contents and incubated at 37° for four days. No growth was observed in cultures made from these tubes after exposure to a temperature of 55°C. or higher. The thermal death point therefore lies between 50° and 55°C.

Resistance toward chemicals

The yeast's resistance toward various chemicals was determined by using a twenty-four-hour lactose broth culture of the organism. This was thoroughly mixed to break up all clumps and masses. Three standardized platinum loops were used for

inoculating the tubes of disinfectants and for transferring the organisms from the disinfectants to the culture media. Sterile litmus milk was used as the culture medium.

The disinfectants employed were: washing soda, calcium hypochlorite, lime, boric acid, cresol and carbolic acid. The vitality of the exposed cultures was determined by incubating

TABLE 2

Resistance towards disinfectants

DISINFECTANT	STRENGTH OF DISIN-FECTANT	MINUTES EXPOSED TO DISINFECTANTS											
		1	3	5	10	15	30	45	60	76	90	150	120
	per cent												
Washing soda	1	+	+	+	+	+	+	+	+	+	+	+	+
Washing soda	2	+	+	+	+	+	+	+	+	+	+	+	+
Washing soda	5	+	+	+	+	+	+	+	+	−	−	−	−
Calcium hypochlorite	1	+	−	−	−	−	−	−	−	−	−	−	−
Calcium hypochlorite	2	−	−	−	−	−	−	−	−	−	−	−	−
Calcium hypochlorite	5	−	−	−	−	−	−	−	−	−	−	−	−
Lime	1	+	−	−	−	−	−	−	−	−	−	−	−
Lime	2	+	−	−	−	−	−	−	−	−	−	−	−
Lime	5	+	−	−	−	−	−	−	−	−	−	−	−
Boric acid	1	+	+	+	+	+	+	+	+	+	+	+	+
Borc acid	2	+	+	+	+	+	+	+	+	+	+	+	+
Boric acid	5	+	+	+	+	+	+	+	+	+	+	+	+
Cresol	0.5	+	+	+	+	+	+	+	+	+	+	+	+
Cresol	1	+	−	−	−	−	−	−	−	−	−	−	−
Cresol	2	−	−	−	−	−	−	−	−	−	−	−	−
Carbolic acid	5	−	−	−	−	−	−	−	−	−	−	−	−

Growth +; no growth −.

the litmus milk tubes for four days at 37° and noting the presence or absence of growth.

The results are noted in table 2.

The results in table 2 indicate that washing soda exhibits little disinfectant action on the yeast. Exposure for two hours to both 1 and 2 per cent solutions failed to kill the organism, while a 5 per cent solution prevented their growth in seventy-five minutes.

Calcium hypochlorite prevents the development of the yeast in 2 and 5 per cent solutions within one minute, while a 1 per cent solution acts similarly in three minutes.

Lime possesses disinfectant properties in 1, 2 and 5 per cent solutions with three minutes' exposure.

Boric acid exhibits no killing powers in strengths of 1, 2 and 5 per cent after two hours.

Cresol in 0.5 per cent solution fails to destroy the yeast in two hours while 1 per cent kills in three minutes and 2 per cent in one minute.

Carbolic acid in 5 per cent solution is likewise efficient within one minute.

Resistance toward desiccation

Data showing the ability of the yeast to withstand desiccation are somewhat limited. However, the results obtained demonstrate it to be very resistant to drying. Soil and alfalfa stems were placed in sterile containers and inoculated with milk cultures of the yeast. The absence of yeasts in the soil and alfalfa used was assured before inoculation. The substances were examined at frequent intervals to note the effect of desiccation. The yeasts were observed after eighty-six days in large numbers in both substances. No further analysis of the materials was made.

CONCLUSIONS

1. A lactose-fermenting yeast is the essential organism in the abnormal fermentation of cream, known as "foamy cream."
2. Raw or sterile cream inoculated with a pure culture of the yeast shows typical foaming characteristics.
3. The optimum temperature for growth is near 37°C. This accounts for the prevalence of foamy cream during hot weather only.
4. The thermal death point of the yeast is near 55°C. for ten minutes.
5. The yeast offers slight resistance toward efficient disinfectants.
6. The organism is quite resistant towards desiccation.

REFERENCES

Beijerinck, M. W. 1889 Die Lactase ein neues Enzym. Centralbl. f. Bakteriol. Abt. I, **6**, 44.

Bochicchio, Nicola 1894 Ueber einen milchzucker Vergärenden und Käseblähungen hervorrufenden neuen Hefepilz. Centralbl. Bakteriol. Abt I, **15**, 546.

Duclaux, E. 1900 Traité de Microbiologie, Paris, 682.

Freudenreich, Ed. V. and Jensen, Orla 1897 Ueber den Einfluss des Naturlabes auf die Reifung des Emmenthalerkäses. Centralbl. f. Bakteriol. Abt. II, **3**, 545.

Grotenfelt, G. 1889 Studien über die Zersetzungen der Milch. Centralbl. f. Bakteriol. Abt. I, **5**, 607.

Harrison, F. C. 1902 Bitter milk and cheese, Centralbl. f. Bakteriol. Abt. II, **9**, 206–225.

Jensen, Orla 1902 Studien über das Ranzigwerden der Butter. Centralbl. f. Bakteriol. Abt. II, **8**, 251.

Jorgensen, Alfred 1911 Microörganisms and Fermentation. Philadelphia, 371.

Lafar, Franz 1911 Technical Mycology, Vol. II, London, 183–209; 288–298.

Maze, P. 1903 Quelques nouvelles races de levures de lactose. Ann. de l'Inst. Past., **17**, II.

Russell, H. L. and Hastings, E. G. 1905 A Swiss cheese trouble caused by a gas forming yeast. Wis. Exp. Station, Bul. No. 128.

STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

VII. THE SUBGROUPS AND GENERA OF THE CHLAMYDOBACTERIALES

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Order II. **Chlamydobacteriales.** *ordo nov.*

Synonyms:

Cladotrichiae Trevisan, 1879, p. 15
Cladotricheen Zopf, 1883, p. 45
Cladothrichacei Schroeter, 1886, p. 173
Cladothricheae Hueppe, 1886, p. 140
Leptotrichacei Schroeter, 1886, p. 170
Crenotricheae DeToni and Trevisan, 1889, p. 925
Chlamydobacteriaceae Migula, 1894, p. 237
Desmobacteriaceae Benecke, 1912, p. 188 in part
Phycobacteriales Engler

Filamentous bacteria, alga-like, typically water forms, frequently sheathed, without true branching although false branching may be present. The sheath is frequently impregnated with iron. Conidia may be developed, but never endospores. Sulphur granules or bacteriopurpurin never present. Mature cells or filaments not motile, not protozoan-like.

The order contains a single family *Chlamydobacteriaceae.*

Family I. **Chlamydobacteriaceae** Migula, 1894, p. 237

The following genera have been included in the family by various authors:

Gaillonella Bory, 1823
Sphaerotilus Kuetzing, 1833, p. 385
Gallionella Ehrenberg, 1838, p. 166
Leptothrix Kuetzing, 1843, p. 198
Didymohelix Griffith, 1853, p. 438
Crenothrix Cohn, 1872, p. 130
Siphonomyxa Billroth, 1874, p. 27
Cladothrix Cohn, 1875, p. 185
Leptotrichia Trevisan, 1879, p. 138, in part
Phragmidiothrix, Engler 1882, p. 19
Kurthia Trevisan, 1885, p. 92
Billetia Trevisan, 1889, p. 11
Detoniella De Toni and Trevisan, 1889, p. 929
Leptotrichella De Toni and Trevisan, 1889, p. 935
Streblothrichia Guignard, 1890, p. 124.
Eucrenothrix Hansgirg, 1891, p. 313
Chlamydothrix Migula, 1900, p. 1030
Clonothrix Schorler, 1904, p. 691.
Spirophyllum Ellis, 1907, p. 516
Nodofolium Ellis, 1910, p. 321.
Leucothrix Oersted, ——, p. 44.

Of these the following will be disregarded because of the inadequate characterization of genus and species: *Billetia, Kurthia, Siphonomyxa, Streblothrichia.*

The following are algal genera to which certain of these organisms were incorrectly assigned: *Gaillonella, Gallionella.*

The following names are invalid because of the prior use of the names for distinct groups.

Spirophyllum Ellis, 1907, p. 516
not *Spirophyllum* Schindler, 1905, p. 82
Cladothrix Cohn, 1875, p. 185
not *Cladothrix* Nuttall, 1849

The following are subgenera: *Eucrenothrix, Leptotrichiella, Leucothrix.*

The following generic names must be considered: *Chlamydothrix, Clonothrix, Crenothrix, Detoniella, Didymohelix, Leptothrix, Leptotrichia, Nodofolium, Phragmidiothrix, Sphaerotilus.*

The following key give the characters which are believed by the writer to differentiate the genera which may be recognized.

Key to the genera of Chlamydobacteriaceae

1. Filaments not usually permanently attached.
   - a. Filaments straight, or at least not twisted......Genus 1. *Leptothrix*
   - b. Filaments twisted..............................Genus 2. *Didymohelix*
2. Filaments attached.
   - a. Filaments unbranched..........................Genus 3. *Crenothrix*
   - b. Filaments show pseudodichotomous or false branching.
     - (1) Swarm cells developed (motile conidia). Usually without a deposit of iron oxid in the sheath................Genus 4. *Sphaerotilus*
     - (2) Spherical, non-motile conidia. Usually with iron oxide. Genus 5. *Clonothrix*

Genus 1. **Leptothrix** Kuetzing, 1843, p. 198

Synonyms:

Chlamydothrix Migula, 1900, p. 1030
Leptotrichia Trevisan, 1879, p. 138
Detoniella Trevisan, 1889, p. 929

Filaments of cylindric colorless cells, with a sheath at first thin and colorless, later thicker, yellow or brown, becoming encrusted with iron oxide. The iron may be dissolved by dilute acid, whereupon the inner cells show up well. Multiplication is through the division and abstriction of cells and motile cylindric swarm cells. Swarm cells sometimes germinate in the sheath giving appearance of branching. Pseudodichotomous branching may occur.

The type species is *Leptothrix ochracea* (Leiblein) Kuetzing.

There has been considerable confusion relative to the appropriate designation of this genus. The name *Leptothrix* was created by Kuetzing for certain forms regarded as algae. The first species named was *L. ochracea,* the *Lyngbya ochracea* of Leiblein. Three other species were also described. *Leptothrix buccalis* an organism from the mouth, was named by Robin in 1852.

These facts have led to the development of three conceptions of the genus as follows:

1. *Leptothrix*. A genus of bacteria with the *L. ochracea* as the type.

2. *Leptothrix*. A genus of bacteria with *L. buccalis* as the type.

3. *Leptothrix*. A genus of algae. In this sense the genus has been recognized by many algologists. However, it may be noted that by recent writers (as in West's British Fresh Water Algae) the genus *Leptothrix* is made a synonym of *Lyngbya*. Many authors include *L. ochracea* with the algae.

Inasmuch as *Leptothrix ochracea* was definitely first named in this genus, it would seem to be entirely appropriate to make it they type of the genus.

It should be noted that this renders *Leptothrix* as applied to organisms such as *L. buccalis* quite invalid.

Genus 2. **Didymohelix** Griffith, 1853, p. 438

Synonyms:

Gaillonella Bory, 1823, in part
Gallionella Ehrenberg, 1838, p. 166, in part
Gloeotila Kuetzing, 1843, p. 245, in part
Spirophyllum ? Ellis, 1907, p. 516
Nodofolium ? Ellis, 1910, p. 321

Filament twisted, simple, or two filaments, twisted together. Young cells colorless, later yellow brown to rust red through deposition of iron. Simple filaments show no division into cells, even when iron is removed with acid and stain applied. Sheath not demonstrable.

The type species is *Didymohelix ferruginea* (Ehr.) Griffiths.

The generic name *Gallionella* was first used by Ehrenberg as a revised spelling of *Gaillonella*, a genus of diatoms created by Bory de St. Vincent. Ehrenberg included several true diatoms in the genus, together with this form, which he erroniously believed contained silicon, and to be a diatom. *Gallionella* is a valid diatom genus (or subgenus of *Meloseira* according to some authors), and should not be used as a generic name for bacteria.

Genus 3. **Crenothrix** Cohn, 1870, p. 130

Filaments unbranched, showing differentiation of base and tip, attached, usually thicker toward the tip. Sheaths plainly visible usually colorless, brownish from iron oxid in old filaments. Cells cyclindric to spherical. Multiplication by non-motile, spherical, conidia; cells dividing in 3 planes to form conidia.

The type (and only) species is *Crenothrix polyspora* Cohn.

Genus 4. **Sphaerotilus** Kuetzing, 1833, p. 385

Synonyms:
Cladothrix Cohn, 1875, p. 185

Attached colorless threads showing false branching, making a psuedodichotomy. Filaments consist of rod or oval cells, surrounded by a thin, firm sheath. Multiplication occurs both by non-motile and motile gonidia, the latter with a clump of flagella near one end.

Sphaerotilus natans Kuetzing is the type.

Genus 5. **Clonothrix** Schorler, 1904, p. 689

Filaments with false dichotomous or irregular branching, attached, with contrast of base and tip, tapering to the tip. Sheath always present, thin on young filaments, later becoming thicker and encrusted with iron or manganese. Multiplication by small non-motile gonidia of spherical form, formed from the disk shaped cells near tip by longitudinal division on rounding up.

The type species is *Clonothrix fusca* Schorler.

REFERENCES

BENECKE, WILHELM 1912 Bau und Leben der Bakterien.

BILLROTH, THEODOR 1874 Coccobacteria septica.

BORY DE ST. VINCENT 1823 Dict. Classique d'hist. nat. according to Ehrenberg.

COHN, FERDINAND 1872 Untersuchungen über Bakterien. Beiträge zur Biologie der Pflanzen. **1** (Heft 1), 127–224.

COHN, FERDINAND 1875 Untersuchungen über Bakterien. II. Beiträge zur Biologie der Pflanzen. **1** (Heft 3): 141–208.

DETONI, J. B. AND TREVISAN, V. 1889 Schizomycetaceae Naeg. in Saccardo's Sylloge Fungorum. **8**, 923–1087.

EHRENBERG, C. G. 1838 Die Infusionsthierchen als vollkommende Organismen. Leipzig.

ELLIS, DAVID. 1907 A Contribution to our Knowledge of the Thread Bacteria. Centralbl. f. Bakteriol. (etc.) Abt. 2. **19**, 516.

ENGLER 1882 Ueber die Pilzvegetation des weissen oder toten Grundes in der Kieler Bucht. Verhandl. des kot. Vereins der Provinz Brandenburg. p. 19.

GRIFFITH, J. W. 1853 On *Gallionella ferruginea*. (Ehrenb.) Annals and Magazine of Natural History. Series 2. **12**, 438.

GUIGNARD, LEON 1890 Sur une nouvelle Bacteriacee marin, le *Streblothrichia Bornetii*. Compt. rend. d Soc. de biol. **42**, 124.

HANSGIRG, ANTON 1891 Ueber die Bacteriaceen Gattung *Phragmidiothrix* Engler und enige Leptothrixarten. Bot. ztg. **49**, 313.

HUEPPE, F. 1886 Die Formen der Bakterien und ihre Beziehungen zu den Gattungen und Arten. Wiesbaden.

KUETZING, F. T. 1833 Linnaea, **8**, 385.

KUETZING, F. T. 1843 Phycologia generalis.

MIGULA, W. 1894 Ueber ein neues System der Bakterien. Arbeiten aus den bakt. Inst. der techn. Hochschule zu Kalrsruhe. **1**, 235–238.

MIGULA, W. 1900 System der Bakterien. Vol. 1, 1897. Vol. 2

OERSTED. De regionibus marinis, p. 44.

SCHORLER. 1904 Beiträge zur Kenntniss der Eisenbakterien. Centralbl. f. Bakteriol. (etc.) Abt. 2. **12**, 691.

SCHROETER, J. 1886 Die Pilze, in Cohns Kryptogamen Flora von Schlesien.

TREVISAN, V. 1879 Prime linee d'introduzione allo studio dei Batterj. italiani. Rendiconti. Reale Istituto Lombardo di Scienze e Lettere IV. Ser. **2**. **12**, 133–151.

TREVISAN, V. 1885 Caratteri di alcuni nuovi generi di Batteriacee. Atti della Accademia Fiso-Medico-Statistica, in Milano. Ser. 4. **3**, 92–107.

TREVISAN, V. 1889 Genera e sp. delle Batteriacee.

ZOPF, W. 1883 Die Spaltpilze.

EARLY INSTRUCTORS IN BACTERIOLOGY IN THE UNITED STATES

ADDENDA

E. G. HASTINGS AND C. B. MORREY

THE WORK OF WILLIAM TRELEASE

The recent paper[1] by Dr. D. H. Bergey on the "Early Instructors in Bacteriology in the United States" is of historic interest. It would seem that it should be made as complete as possible. The name of Dr. William Trelease is mentioned in connection with the development of bacteriology at the University of Wisconsin. No facts, however, are given as to the importance of his work in this connection.

Dr. Trelease came to the University of Wisconsin in 1881, leaving in 1885 to assume charge of the Missouri Botanical Gardens at St. Louis. In 1884 Dr. Trelease presented to the Academic Council of Harvard University as a doctor's thesis the results of some work he had done in the botanical laboratories at the University of Wisconsin. This work was published in the Studies from the Biological Laboratory of the Johns Hopkins University, Vol. III, No. 4, 1885 under the title "Observations on Several Zoogloeae and Related Forms."

Dr. L. H. Pammel, Wisconsin 1885, who is mentioned by Dr. Bergey as one of the pioneer instructors in bacteriology, received his first instruction in the subject from Dr. Trelease. Dr. Pammel states in a personal letter that Dr. Trelease introduced some work with the bacteria into his course in cryptogamic botany in the first semester of 1882–1883. Cultures were made on potato. The organisms of the mouth were studied, and the students were required to read Dr. Burrill's paper on "Bacteria," which is mentioned by Dr. Bergey. The work of Pasteur, Tyndall, Koch, Cohn, and others was discussed.

[1] J. Bact. 2, 595.

The first bacteriological apparatus was ordered by the University from Germany in 1885 at the request of Dr. Trelease. It did not arrive until after his departure, and it remained for Dr. E. A. Birge to develop formal courses in bacteriology at the University of Wisconsin. Dr. Trelease was, however, one of the first to give instruction in bacteriology in this country.

E. G. HASTINGS.

BACTERIOLOGY AT OHIO STATE UNIVERSITY

In the November, 1917, number of the Journal of Bacteriology there is an article by Dr. David H. Bergey on "Early Instructors in Bacteriology in the United States." I should like to add a few words concerning the teaching of bacteriology at the Ohio State University.

In the fall of 1885 Dr. H. J. Detmers came to the University as professor of veterinary surgery. He had previously been in the Bureau of Animal Industry associated with Dr. Salmon and also at the University of Illinois. He began doing bacteriological work as soon as he was established and the catalogue for 1886 lists a course in bacteriology for the spring term of one hour per week for senior veterinary students. By 1890 this had grown to a three hour course. Dr. Detmers did some research work on hog cholera during these years, and one of his students presented a paper at the Pittsburgh meeting of the American Microscopical Society, September 1, 1887, on the "Bacteriology of Foot Rot in Sheep," which paper was favorably commented upon in a number of journals throughout the country.

Not later than 1888 though I have not the exact date at hand, courses in bacteriology were offered to medical students in Starling Medical College, now a part of the University.

C. B. MORREY.

STUDIES RELATIVE TO THE APPARENT CLOSE RELATIONSHIP BETWEEN BACT. PERTUSSIS AND B. BRONCHISEPTICUS[1]

II. COMPLEMENT FIXATION TESTS

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In a previous article (Ferry and Noble, 1918) we have described the cultural, agglutination and absorption reactions between *Bact. pertussis* and *B. bronchisepticus* and have shown that, while the two organisms are distinct, they are apparently somewhat closely related. The most striking characteristics of the organisms, according to the serological reactions, were shown to be the ability of *B. bronchisepticus* to produce an immune serum that would agglutinate both the *B. bronchisepticus* and *Bact. pertussis* antigens and the ability of *Bact. pertussis* to produce an immune serum that would agglutinate only the homologous antigen. The absorption reaction showed that the *B. bronchisepticus* antigen would absorb from the antibronchisepticus serum (a serum that contained agglutinins for both organisms) only the *B. bronchisepticus* agglutinin (the major agglutinin) leaving intact the agglutinin for *Bact. pertussis* (the minor agglutinin). This minor agglutinin could only be absorbed by the *Bact. pertussis* antigen. This type of an agglutinin was termed by the authors a "transitive" agglutinin.

The present investigation was undertaken to confirm the work of the previous paper through complement fixation tests and to determine, if possible, the value of this test in differentiating between the two organisms.

Strains used. At first a large number of strains of each organism were used, the same strains as those worked with in the pre-

[1] Presented at Eighteenth Annual Meeting of the Society of American Bacteriologists, New Haven, Conn., December 27–29, 1916.

vious experiments already described, but as it was found that all strains of the same organism gave similar reactions it was deemed advisable to cut down the number to three of each in order to save time. Of the *B. bronchisepticus*, no. 36 (dog), no. 123 (monkey) and human strains were tested; and of *Bact. pertussis*, no. 0363 (Bordet), no. 109 and no. 248 (Povitzky).

Technic. For the volume of the complement fixation tests it was found more satisfactory to use 2 cc. than 5 cc. as advised for the Wassermann test or 0.5 cc. suggested by Olmstead and Povitzky in a serological comparison of the Bordet-Gengou bacillus and hemoglobinophilic bacilli. The hemolytic system was composed of sheep cells in a 2 per cent suspension, guinea-pig complement in a 1 to 10 dilution and rabbit amboceptor in 1 to 1500 dilution. Complement titration was made by using 0.1 cc. of amboceptor and varying amounts of complement.

In determining the relationship of the various strains two units of both amboceptor and complement were employed. All titrations were incubated one hour before and one hour after the addition of the sensitized cells, at 32°C. The dilution was chosen in which complete hemolysis was produced, readings being made at the end of the hour's incubation. All serum was inactivated by heating in water bath one half hour at 56°C.

The antigen was titrated by mixing it in varying amounts with one unit of the hemolytic system.

Preparation of antigen. After trying out several methods of antigen preparation it was finally determined that filtered autolysates gave the most stable and satisfactory products.

The antigens were prepared as follows: The organisms were grown on agar for forty-eight hours at 37.5°C., then taken off and suspended in distilled water and shaken for forty-eight hours in a mechanical shaker. This suspension was then heated at 56°C. for one-half hour, incubated twelve hours, after which enough sodium chloride and formalin was added to make an 0.85 per cent and 0.5 per cent solution respectively. Filtration was carried on through asbestos.

Preparation of immune serum. The same serums were used for this work as for the previous experiments, a description of which has already been given.

The results of the complement fixation tests may be seen in the following table:

ANTIGENS	ANTISERUMS					
	B. bronchisepticus (dog) no. 36	B. bronchisepticus (monkey) no. 123	B. bronchisepticus (human)	Bact. pertussis no. 0363	Bact. pertussis no. 109	Bact. pertussis no. 248
B. bronchisepticus (dog) no. 123	+	+	+	–	–	–
B. bronchisepticus (monkey) no. 123	+	+	+	+	+	+
B. bronchisepticus (human)	+	+	+	+	+	+
Bact. pertussis no. 0363	+	+	+	+	+	+
Bact. pertussis no. 109	+	+	+	+	+	+
Bact. pertussis no. 248	+	+	+	+	+	+

+ Denotes complete inhibition of hemolysis.
– Denotes incomplete or no inhibition of hemolysis.

It was found in a large majority of the tests as represented in the chart, which is a composite, that the *B. bronchisepticus* immune serum bound the complement in the presence of both the bronchisepticus and pertussis antigens, while the *B. pertussis* immune serum bound the complement in the presence of the homologous antigen and also the human and monkey strains of *B. bronchisepticus.* It did not bind the complement in the pressence of a dog strain of *B. bronchisepticus.*

SUMMARY

1. *B. bronchisepticus* immune serum bound the complement in the presence of both *B. bronchisepticus* and *Bact. pertussis* antigen.

2. *Bact. pertussis* immune serum bound the complement in the presence of *Bact. pertussis* antigen and *B. bronchisepticus* antigen of both human and monkey origin but not of dog origin.

3. The complement fixation test is not a reliable method of differentiating between the two organisms in question.

4. Bacterial autolysates were found to be the most stable and satisfactory antigens.

5. The complement fixation test was found to corroborate, in most respects, the agglutinin reactions reported in a previous paper.

REFERENCES

Ferry, N. S. and Noble, Arlyle 1918 Studies relative to the apparent close relationship between Bact. pertussis and B. bronchisepticus. I. Cultural, agglutination and absorption reactions. J. Bact., Balt., **3**, 193.

THE OCCURRENCE OF DIFFERENT TYPES OF THE COLON-AEROGENES GROUP IN WATER[1]

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Recent work on the colon-aerogenes group of bacteria (Levine, 1916; Rogers, Clark and Davis, 1914; Rogers, Clark and Evans, 1914 and 1915; Winslow and Kligler, 1916) has confirmed and amplified the view held by most bacteriologists that the group includes two distinct types or species represented by *B. coli* and *B. aerogenes*. Although the distinction between the two types has not been very clearly defined, the former has been considered as the predominant organism of the feces of warm blooded animals.

The work cited has demonstrated that there is a real difference between the two colon types and that this difference can be detected by certain simple laboratory tests.

The *B. coli* type is characterized by the production of almost exactly equal volumes of hydrogen and carbon dioxide in the anaerobic fermentation of glucose, the failure to give the Voges-Proskauer reaction, the formation of indol from tryptophane and the comparatively low fermentative ability of a greater part of the cultures.

The *B. aerogenes* type, on the other hand produces an excess of carbon dioxide over hydrogen in the anaerobic fermentation

[1] Published by permission of the Secretary of Agriculture.

The writer wishes to acknowledge his indebtedness to his colleagues, particularly to Dr. William M. Clark for assistance and for many suggestions.

Since this paper was written, the writer has had the privilege of reading two manuscripts by Winslow and Cohen. They cover much the same ground as this paper and the results and conclusions drawn are substantially in accord with those given here.

of glucose, gives a positive Voges-Proskauer reaction, usually fails to form indol from tryptophane and ferments many carbohydrates and alcohols. The tendency to produce rapidly a hydrogen ion concentration which will inhibit alkali formation and consequently any subsequent reduction of the acidity has been made use of by Clark (1915) to distinguish the *B. coli* type from *B. aerogenes* by the methyl red test.

The *B. coli* type is found abundantly in bovine feces while the *B. aerogenes* type is very rare. In human feces the latter type is more common, but apparently subject to considerable variation. These results agree with the conclusions of some of the earlier workers, particularly Clemesha (1912) who states that sucrose +, dulcite −, adonite −, inuline −, V. and P. + organisms are rare in feces (about 1 to 2 per cent). These characters agree with those of the typical high ratio or *B. aerogenes* cultures found in feces.

The work in our laboratories on the colon-aerogenes bacteria of human feces also shows a possible line of demarcation between two varieties of the *B. aerogenes* type. All of the *B. aerogenes* cultures isolated from human feces fermented adonite. Many formed indol, 60 per cent fermented starch and in other ways showed a higher fermentative ability than *B. aerogenes* cultures from other sources.

On the other hand the collection of cultures from grains, consisting very largely of *B. aerogenes* cultures, contained very few adonite fermenters. Those that did ferment adonite agreed very closely with the adonite fermenters of feces while the non fermenters were less active in the formation of indol and in the fermentation of sucrose, raffinose, starch, mannite and glycerine. Disregarding, for the present, any question of the value of the differences enumerated for purposes of classification we believe that it is safe to use them as an indication of the original source of the culture.

It does not necessarily follow that the only source of *B. coli* or the adonite fermenting *B. aerogenes* is the intestine of warm blooded animals but the relative frequency with which they occur there makes it highly probable that any culture with these characters, wherever found, came originally from this habitat.

The value from a sanitary standpoint of an ability to distinguish between different types of colon bacilli rests on the answer which we can give to certain questions. First we are concerned with possibilities of any type of colon bacillus existing normally outside of the digestive tract of animals. If a colon culture of any kind is isolated from water does it necessarily indicate fecal contamination or must we distinguish between those of fecal and those of nonfecal origin?

Secondly what is the fate of the fecal colon bacillus in water? Does it multiply or decrease rapidly? Does the typical fecal colon bacillus become attenuated so that its characters change and it can no longer be recognized?

The first question is partially answered by the results of our comparison of the characters of fecal colon with those from other sources, particularly from grains. The marked variation of the grain cultures from the fecal types indicates that water may receive bacteria of the colon-aerogenes group from other than fecal sources.

Some light has been thrown on the second problem by the observations of Clemesha (1912). He found that the different types of colon did not decrease in water at an equal rate and that in some cases there was an actual increase.

The characters used by Clemesha differ somewhat from those which we have used and only rough comparisons can be made. We have studied this problem by observations on water held in bottles, and on cultures in parchment sacs in running water, by watching the progressive changes in streams and by examination of individual samples from various sources.

While it is realized that the results are not sufficiently comprehensive to permit any positive conclusions it is hoped that they may present something of value.

CHANGES IN SAMPLES HELD IN BOTTLES

In considering bacterial changes in water samples held in bottles, one should remember that the conditions are not necessarily comparable with those obtained in streams or reservoirs.

The concentrations, both of food-stuffs and products of metabolism, are likely to be much greater in the bottle and the results may vary accordingly.

A number of bottles containing 100 cc. of sterile water to which a loopful of human feces had been added were left in a 20° incubator for several months.

These bottles were used for another purpose and from the earlier plates made only a few cultures were isolated. Later a total colon count was made and a considerable number of cultures isolated. In table 1 are given the results from a bottle which was held at 20° for 278 days.

TABLE 1

Changes in colon content in water held at 20°

AGE	COLON GROUP PER CUBIC CENTIMETER	CULTURES ISOLATED		P_H LIMIT OF B. COLI CULTURES						INDOL BY B. COLI CULTURES	
		B. aerogenes	B. coli	4.8	4.9	5.0	5.2	5.4	5.6	+	−
days											
0		0	3	1	2					3	0
17		1	8	2	5	1				8	0
31	4,700,000	1	6	2	3	1				5	0
47	2,100,000	0	9			9				9	0
78	3,530,000	0	12			11	1			11	1
109	2,530,000	4	46								
137	2,090,000	7	58								
160		8	32				23	9		24	1
199	174,000	11	31	2		26	1	1	1	23	8
278	38,750	39	1				1				

The total number of colon forms decreased slowly in this period. At first there was a great preponderance of *B. coli* over *B. aerogenes*. Even after 100 days there were ten times as many *B. coli* as *B. aerogenes*. But this relation was gradually changed until at 278 days there was only one *B. coli* in the 40 cultures isolated.

Another change which may be of some significance is found in the apparent decrease in limiting hydrogen ion concentration reached by the *B. coli* cultures. The P_H value of 5.0 to 4.8 observed in the first cultures isolated is normal for freshly iso-

lated *B. coli* cultures. In the *B. coli* cultures isolated after they had been exposed for a long time to the unfavorable conditions of the water there was some evidence of lowered vitality, in the large numbers of cultures which carried the hydrogen ion concentration[2] to 5, 5.2 or 5.4. What may possibly be another evidence of attenuation is seen in the number of *B. coli* cultures which fail to form indol among those isolated after prolonged exposure to the water.

Many water bacteriologists consider weak gas formation an indication of "attenuation" and therefore evidence of a remote contamination.

This view is supported by some observations on six cultures of the *B. aerogenes* type isolated from this bottle of water. Three of these cultures were isolated when the sample was 47 days old and three at 278 days. All of these cultures gave a normal fermentation in glucose. The three cultures isolated at 47 days produced from 10 cc. of lactose broth approximately 2.7 cc. of gas consisting of carbon dioxide and hydrogen in the ratio of 1.53, 1.54 and 1.48 respectively. This is the normal fermentation for the *B. aerogenes* type. The three cultures isolated at 278 days gave, under similar conditions, 3.77, 3.78 and 3.77 cc. of gas with a ratio of 0.59, 0.56 and 0.51 respectively. These cultures were of course, not identical with those isolated at the earlier date and the evidence of "attenuation" is thus purely circumstantial.

However all of these observations considered together point to a slow change, which may be described as a loss of function, in colon cultures held for a long time in water. This change was perceptible only after the culture had been in water for many weeks.

These results may seem at variance with conclusion reached by Browne who studied changes in bottled water under similar conditions. The conflict is probably only apparent.

Browne's sample was held only 73 days and in that period there was little evidence of change in our sample. Moreover

[2] It should be remembered that the numbers on Sorensen's scale run *inversely as the hydrogen ion concentration.*

Browne used the MacConkey-Jackson system of classification which is merely the possible arrangements of plus and minus signs under sucrose and dulcite and has no relation to the varieties arranged by nature.

CULTURES HELD IN WATER IN PERMEABLE SACS

Conditions more nearly approximating those found when sewage is emptied into streams were obtained by holding cultures or fecal matter in parchment sacs suspended in running water. This was repeated several times both in the laboratory and in small streams, but nearly every experiment came to an untimely end through overheating, freshets or other causes before very complete results were obtained.

TABLE 2

Changes in colon bacteria in running water

AGE	COLON GROUP PER CUBIC CENTIMETER	RATIO B. AEROGENES TO B. COLI
days		
0	190,000	1:2.3
1	130,000	1:4
2	19,000	1:3.3
3	9,000	1:1.2
4	20	1:1.1
7	30	1:0.11

In all cases in which the temperature of the water was relatively high there was an increase in the colon bacteria of both types.

Table 2 shows the results obtained by holding a small amount of dilute sewage in a parchment sac in running water. The sac was made by folding parchment paper around a bottle from which the bottom had been removed. This bottle properly protected, was held in running tap water at a temperature of 16 to 20.6° C.

There was no increase in numbers observed but otherwise these results agreed with those obtained on other sacs under similar conditions. The total number decreased far more rapidly than was the case in the bottle held in the incubator at 20° and

the change in the ratio of *B. aerogenes* to *B. coli* was correspondingly abrupt. The initial determinations showed three or four times as many *B. coli* as *B. aerogenes* but at 7 days there were ten times as many *B. aerogenes* as *B. coli*.

There is a possibility that on account of imperfections in the parchment there was a mechanical loss of bacteria. The results obtained were however consistent and in accord with those reported by other investigators. Moreover it is very improbable that a mechanical loss would have resulted in the relative changes observed in the abundance of the two species.

RELATIVE CHANGES IN COLON-AEROGENES BACTERIA IN POLLUTED STREAMS

It is difficult to even approximate the total number of colon-aerogenes bacteria in a polluted stream but the relative number of *B. coli* and *B. aerogenes* may be obtained with a fair degree of correctness by isolating a considerable number of colonies and determining the group to which they belong by the methyl red test.

This was done on two representative streams. The samples, which were collected by following down the stream in an automobile, were held in ice water and taken to the laboratory at once. They were plated on asparagin agar, a medium on which colon types grow well, but which is not favorable to many other bacteria particularly the streptococci.

One of these streams was Wolf Creek, a rather sluggish stream originating in swamps and flowing through Grove City, a town of about 4000 inhabitants. On its entire course through the town it is rendered stagnant by a dam and is polluted by houses and stables on its banks. At the lower limits of the town it receives the untreated city sewage. Below Grove City it flows through a partly wooded farming country and for 15 miles receives no sewage. Samples were collected at approximately 2 mile intervals and a number of colon cultures obtained from each by direct plating. There is of course an element of chance in picking cultures in this way but the results shown in table 3 are probably fairly representative.

Above the city where there was no sewage pollution all of the cultures isolated were *B. aerogenes*. After flowing through the town, by which it was badly polluted, there was a preponderance of cultures of the *B. coli* type. Two miles below the city sewer there was only 1 *B. aerogenes* culture in 11 isolated. This ratio changed rapidly however, and *B. aerogenes* soon outnumbered *B. coli* though the latter type was still present ten miles below the sewer.

Rock Creek, the second stream investigated, runs into the Potomac river between Washington and Georgetown. It is not so well adapted to this study as Wolf Creek because it receives sewage at various points and the self purification cannot be so satisfactorily observed. In its upper course it flows through an agricultural country and receives no direct sewage. A few miles above the District of Columbia line the untreated sewage of the village of Kensington is emptied into the stream. There are probably some private sewers before it enters Rock Creek Park in which it is protected from contamination with the exception of two small tributaries, Broad Branch and Piney Branch, both of which are evidently polluted.

TABLE 3

Relative numbers of B. aerogenes and B. coli in Wolf Creek

SAMPLE NO.	MILES	POLLUTION	CULTURES ISOLATED	RATIO B. AEROGENES TO B. COLI
1	0		7	All aerogenes
2	1.1	Private sewers	15	1.1: 1
3	1.1	City sewer	18	0.8: 1
4	3.1		11	0.1: 1
5	5.1		20	9: 1
6	7.1		10	All aerogenes
7	9.1		9	3.5: 1
8	11.3		5	4: 1

The results of the study of Rock Creek are given in table 4. At a point about 10 miles above the district line a sample was taken from which 10 cultures were isolated. All of these were *B. aerogenes*. Two and one-half miles below where the stream passes the small village of Garrett Park nearly one-half of the

cultures isolated were of the *B. coli* type. From a sample taken immediately above the mouth of the Kensington sewer, 3 *B. coli* and 13 *B. aerogenes* cultures, only 2 of which fermented adonite were isolated. A few yards below over half of the cultures isolated were of the *B. coli* type. Of the 13 *B. aerogenes* cultures isolated, 7 fermented adonite which we may assume indicated fecal origin. The fermentation tubes made in the usual way and incubated at 37° did not show a greater number of lactose fermenters immediately below the Kensington sewer than just

TABLE 4

Relative numbers of B. aerogenes and B. coli in Rock Creek

SAMPLE NO.	MILES	POLLUTION	HIGHEST DILUTION SHOWING GAS		CULTURES ISOLATED	RATIO OF B. AEROGENES TO B. COLI	FERMENTATION OF ADONITE BY B. AEROGENES CULTURES	
			24 h.	48 h.			+	−
			cc.	cc.				
1	0		1.0	0.1	10	All aerogenes		
2	2.5	Village without sewers	0.1	0.1	13	1.1 : 1.0		
3	7		0.01	0.01	16	4.3 : 1.0	2	11
4	7	Kensington sewer	0.01	0.01	28	0.86 : 1.0	7	6
5	9		0.1	0.01	22	1.0 : 1.0	4	7
6	10.7		0.01	0.01	22	1.0 : 1.0	5	5
7	12.7	Broad Branch	0.1	0.1	17	1.4 : 1.0		
8	14.5	Piney Branch Zoological Park	0.1	0.1	14	1.0 : 1.0		
9	15.7		0.001	0.001	24	1.0 : 1.1		

above it; that is there was gas in 0.01 cc. dilution but none in the 0.001 cc. dilution in each case. Accurate counts could not be made from the plates but they indicated a great increase at this point.

Samples taken at lower points on Rock Creek showed a slight increase in the proportion of *B. aerogenes* but below the mouth of Broad Branch and Piney Branch bacteriological conditions as shown by these tests were nearly as bad as just below the Kensington sewer. This is probably due to a badly polluted condition in Piney Branch.

THE EXAMINATION OF INDIVIDUAL SAMPLES OF SURFACE WATER

In the course of this work about 30 samples of water from a great variety of sources have been examined. These have included samples from grossly polluted streams such as Rock Creek and the Anacostia river and from springs in the Maine woods in which the chance of pollution was remote. With a few exceptions bacteria of the colon-aerogenes type were isolated from these samples without difficulty. The exceptions were a Maine lake without pollution except from a few camps on the shores, a small stream flowing into this lake and which at no point was near a habitation or a highway, a spring flowing out

TABLE 5

Comparison of cultures from grain, water and feces

SOURCE OF CULTURES	TOTAL CULTURES	B. COLI TYPE	B. AEROGENES TYPE	B. CLOACAE TYPE	B. AEROGENES TYPE				B. CLOACAE TYPE				AVERAGE GELATIN LIQUEFICATION
					Indol	Mannite	Dulcite	Adonite	Indol	Mannite	Dulcite	Adonite	
					per cent +	*per cent* +	*per cent* +	*per cent* +	*per cent* +	*per cent* +	*per cent* +	*per cent* +	*mm.*
Grain....	159	8	111	40	7.20	20.72	16.21	12.61	0	97.5	100	0	5
Water....	134	54	67	13	30.98	91.04	23.88	71.21	15.38	84.62	15.38	58.33	16
Feces.....	177	131	46	0	21.74	100.0	21.74	100.0					

of the gravel on the shore of the lake and a well protected spring in Rock Creek Park, Washington. In all other cases at least 2 or 3 colon cultures were obtained by direct plating.

A total of 134 cultures were isolated from these samples. In table 5 the characters of these cultures as a group are compared with those from grains and from human feces. There will be noticed a general tendency for the water cultures to agree with those of fecal origin rather than with those isolated from grains.

In making this comparison it should be remembered that the grain cultures included some which were very probably of fecal origin while the water cultures included some evidently not of fecal origin. The 12 per cent of the *B. aerogenes* cultures from

grains which fermented adonite had all the characters of the *B. aerogenes* cultures from feces. If these tabulations should be made on this basis, it would be found that the cultures of the fecal *B. aerogenes* type from water would agree very closely with those from feces while they would be quite distinct from the grain cultures. There is a decided difference in the characteristics of the liquefying cultures from grains and from feces but in the light of our present knowledge of this sub-group, it would be unsafe to make any definite deductions from these data. Greenfield, (1916) found that of 405 cultures from ground and surface waters 70 per cent were of the *B. coli* type as indicated by the methyl red and Voges-Proskauer tests.

More light can be thrown on the value of a qualitative examination on the colon bacteria by a study of the results from individual samples. Space will not permit a consideration of all the samples but a few representative ones are given.

No. 21. Rock Creek. This is a polluted stream previously described. Two samples were taken from which 13 cultures were isolated. Eleven of these were high ratio, gelatin −, indol −, adonite +, dulcite −, sucrose and salicin +. One differed from these in being indol + and dulcite +. The characters of these cultures agreed very closely with those of the high ratio cultures isolated from human feces.

In view of the results obtained in the survey of Rock Creek it is rather surprising that no *B. coli* cultures were obtained from these samples. The samples from which *B. coli* were isolated were taken about a year later than those giving all *B. aerogenes*. The disappearance of the *B. coli* type may be looked upon as evidence of self purification and in this the time element is an important factor. The rate of flow which varies greatly in a small stream has a direct influence on the time for which sewage is exposed to purifying influences before it reaches a given point. There is also a possibility that the pollution in the lower part of Rock Creek may have become materially increased after the first samples were taken.

No. 10. The Potomac River. The pollution of this river has been very thoroughly studied (Cumming, 1915). The principal source of pollution is the city of Cumberland about 180 miles above Washington. Sewage is emptied into the river or its tributaries at other points nearer Washington but, considering the volume of water flowing in the river they are relatively unimportant.

Ten cultures were obtained from two samples collected near Washington when the river was in normal flow. These included three cultures of the *B. coli* type which were typical in every way except that they had a hydrogen ion limit of 5.2 to 5.4. In this regard they corresponded to the cultures held in water many weeks rather than with freshly isolated fecal cultures.

The 7 *B. aerogenes* cultures included 5 which fermented adonite and starch and otherwise agreed with the fecal type.

No. 30. Anacostia River. One sample was taken at the bridge below Bladensburg when the flow was above normal. This stream is polluted by the sewage of Hyattsville and other smaller villages. All of the nine cultures were of the *B. coli* type and had a hydrogen ion limit of 4.8 to 5.3.

These results indicate a high pollution of recent origin, comparing as they do with results obtained a short distance below the sewer in Rock Creek and Wolf Creek.

No. 28. Pimmit Run. This is a relatively small stream flowing into the Potomac at Chain Bridge. It probably receives no direct sewage but flows through an agricultural country from which it is contaminated by surface wash. No *B. coli* cultures were obtained from the one sample examined but of the 8 *B. aerogenes* cultures isolated 6 fermented adonite and starch and were probably of fecal origin. One fermented starch but not adonite and one fermented neither adonite nor starch.

No. 26. Spring near Chain Bridge. This spring is in a rather sparsely settled suburban district and was carefully protected from surface contamination by tiles and stone work. The source of the water was not evident and the possibility of contamination was a matter of conjecture.

The houses in the vicinity were, for the most part connected with sewers. Five *B. aerogenes* cultures were isolated, all of which fermented adonite and starch and were therefore of the fecal type.

No. 11. Spring near Little Falls. This spring at a camp on the Virginia shore of the Potomac was carefully protected by stone work. The shore is wooded for nearly a half mile from the river. The camps in the vicinity are occupied at irregular intervals and there are no houses within a mile.

The spring flows from gravel at the foot of an abrupt rocky hill and so far as an examination of the surroundings shows there is no reason to expect contamination. Of the 5 cultures isolated 3 were *B. coli* and two were *B. aerogenes* fermenting adonite and starch.

No. 6. Davis Brook. This is a very small stream in the Maine woods. About 1 mile above the point where the sample was taken it flows from a small but very deep pond fed by submerged springs. On the upper end of this pond is a summer camp. Considering the very small flow from the pond there is only a remote probability of any contamination from this camp affecting the stream.

One of the 4 cultures isolated was of the *B. coli* type. Two of the *B. aerogenes* cultures did not ferment adonite or starch and one fermented starch but the fermentation of adonite was not determined.

Lactose broth tubes inoculated with 1 cc. of water gave small amounts of gas in forty-eight hours.

No. 5. Small Brook. This is a very small stream flowing through dense woods except that at about a mile above where the sample was taken it crosses a highway.

Lactose broth inoculated with 10 cc. of water gave a small amount of gas in forty-eight hours. Of the 4 gas forming cultures isolated 2 were of the *B. coli* type, one was a high ratio liquefier and one was a *B. aerogenes* which failed to ferment adonite.

CONCLUSIONS

Through its greater resistance to the unfavorable conditions found in water the *B. aerogenes* type is able to survive longer than *B. coli*. This was apparent in the water held in bottles, in the sewage held in parchment sacs, in running water and in polluted streams. From this we may draw the inference that water near the source of pollution should contain a greater proportion of *B. coli* to *B. aerogenes* than after the processes of self purification have had an opportunity to act. This was found to be the case in two sewage polluted streams. In each case the gas forming bacteria isolated above the source of pollution consisted largely of *B. aerogenes* cultures, while immediately below the sewers a majority of the cultures isolated were of the *B. coli* type.

At lower points on the streams the proportion of *B. aerogenes* increased again. In the only case in which suitable determinations were made it was found that a similar relation existed between the fecal and nonfecal types of *B. aerogenes*.

The assumption that the two types, *B. coli* and the fecal *B. aerogenes*, are distinctively fecal organisms without other habitat may make it difficult to explain their occurrence in certain samples of water in which the chances of contamination seem very remote. We have isolated both the *B. coli* type and the fecal *B. aerogenes* type from water in which the chances of pollution from dwellings or wash from farm lands is almost completely excluded. In some cases the fact that all of the cultures isolated belonged to one or the other of the two fecal types would point to a source of contamination not found by physical examination of the surroundings.

In no case was the possibility of contamination by animals completely excluded. This is especially true of the springs and brooks in the Maine woods. Deer and moose frequent water courses in the warm months and there are a number of kinds of small animals which make their homes along the banks.

Even a protected spring may be exposed to the visits of squirrels and similar animals. It is possible that the occasional colon bacillus of the fecal type found in waters presumably free from pollution may be accounted for in this way.

The possibility of fish as a source of intestinal bacteria in water is suggested by the work of Browne, (Browne 1917), who found *B. coli* in the intestinal tract of 39.8 per cent of scup examined. The feeding habits of the fish may determine the presence or absence of colon-aerogenes bacteria in its digestive tract but in no case is it likely that fish would account for more than occasional cultures.

There is also a possibility that the digestive tract of animals is not the only source of the so called fecal type of colon. At the present time there is little or no evidence that this is the case. It is true that some of our water cultures were not true to the fecal type and therefore might suggest a different variety or source. These differences were very slight, consisting for the most part in failure to form indol or in a hydrogen ion limit slightly lower than that of the typical culture.

Whatever the final conclusion may be in regard to the occurrence of these occasional cultures the fact remains that there

are two types of the colon-aerogenes group which occur in fecal matter in large numbers. While it is possible that they may also live in the soil or other material from which they may be carried to water, their presence in water is strong presumptive evidence that the water was polluted with fecal matter. One of these types has certain distinctive characters which render identification easy; the other type is not so well marked but may be identified with reasonable certainty and without great difficulty.

In one way the recent contributions to the knowledge of the colon-aerogenes group has not changed the methods of water bacteriology. The presence of any particular kind of bacteria in water is merely an indication of the existence of certain conditions and the bacteriologist must weigh all the available evidence on the basis of his experience and make his decision accordingly. However, the method which we now have of separating the colon-aerogenes group into varieties which have a very definite relation to habitat should be of material assistance in forming an opinion of the potability of a water. The value of this ability to separate the varieties of the colon-aerogenes group is much more evident if a sufficient number of cultures can be isolated from each sample to establish the relative numbers of the different types. This, we believe will prove to be of much greater value than the mere determination of the presence of colon or of any one variety of colon.

REFERENCES

Browne, William W. 1915 Predominance among the members of the bacillus coli group in artificially stored water. J. Infect. Dis. **17**, 72–78.

Browne, William W. 1917 The presence of the Bacillus coli group and the Bacillus welchii group in the intestinal tract of fish (Stenomus chrysops). Abs. Bact. **1**, 55–56.

Clark, William Mansfield 1915 The final hydrogen ion concentrations of cultures of Bacillus coli. J. Biol. Chem., **22**, 87–98.

Clemesha, W. W. 1912 Bacteriology of surface waters in the tropics. London.

Cumming, Hugh S. 1916 Investigation of the pollution and sanitary conditions of the Potomac watershed. U. S. Treasury Dept., Hygienic Laboratory bull. 104.

Greenfield, Myrtle. 1916 The presence of soil and fecal strains of organisms of the colon-aerogenes group in the waters of Kansas. J. Infect. Dis. **19**, 647–651.

Levine, Max. 1916 Acid-production and other characters of Bacillus-coli-like bacteria from feces and sewage. J. Infect. Dis., **19**, 773–805.

Rogers, L. A., Clark, W. M., and Davis, B. J. 1914 The colon group of bacteria. J. Infect. Dis., **14**, 411–475.

Rogers, L. A., Clark, W. M., and Evans, A. C. 1914 The characteristics of bacteria of the colon type found in bovine feces. J. Infect. Dis., **15**, 99–123.

Rogers, L. A., Clark, W. M., and Evans, A. C. 1915 The characteristics of bacteria of the colon type occurring on grains. J. Infect. Dis., **17**, 137–159.

Winslow, C.-E. A., and Kligler, I. J. 1916 Studies on the classification of the colon-typhoid group. J. Bact., **1**, 81.

THE ELIMINATION OF SPURIOUS PRESUMPTIVE TESTS FOR B. COLI IN WATER BY THE USE OF GENTIAN VIOLET

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The bacteriological criterion of potable water is the *absence* of *B. coli* and its close allies,—aerobic, Gram-negative, non-sporulating bacilli or coccobacilli which produce acid and usually gas in media containing lactose, and which do not liquefy gelatin.[1] The best test for purity of water, according to standard methods (1917) is a negative presumptive test, i.e., failure of gas formation in lactose broth; water, which in quantities of 10 cc. or more, does not produce gas, may be unreservedly accepted for domestic use. Even if 1 cc. of a given supply yields a negative result, though colon bacilli are present in larger quantities, the water should probably be regarded as merely suspicious. Only the extreme difficulty of ascertaining the presence of the various intestinal pathogens makes us rely upon the more actively fermenting colon bacillus as a criterion of potential pollution.

While a negative result permits very definite statements as to the purity of the sample, a positive result, i.e., the formation of gas in the presumptive test, does not necessarily condemn the water. Even if the gas produced be proven due to *B. coli* the water may be quite safe if no pathogenic organisms have entered the water along with the colon bacillus. Thus the colon bacilli may have had their origin in perfectly healthy human beings free from disease producing micro-organisms, in

[1] In this paper the term *B. coli* is used to include all members of the colon groups as above defined—*B. acidi-lactici*, *B. aerogenes*, *B. communior*, *B. communis*, and their subvarieties.

birds or lower mammals whose intestinal diseases are not ordinarily transferable to man, or even in certain non animal sources, e.g., grains. Methods have recently been described which promise some degree of differentiation of certain of these forms; the literature was fully reviewed by Winslow (1916) and need not be repeated in detail here. While fully appreciative of the refinements in interpretation which the work of Rogers, Clark and Davis (1914), Rogers, Clark and Evans (1915), Clark and Lubs (1915), and others makes possible, we are convinced that an equally important problem concerns the use to be made of the presumptive test. We are not willing to discard this test as Professor Winslow advises in case of water of fair quality; indeed we feel that inasmuch as negative presumptive tests are most significant it is in just this type of water, even more than in badly polluted water, that the presumptive test is of most value. We fully agree however as to the need of the colon confirmatory test where the presumptive test is positive. Finally, and most important, the finding of *B. coli* in water should properly be interpreted *only* as an indication of the absolute necessity of a sanitary survey to locate, identify, and, if possible, eradicate the source of pollution.

In a varying proportion of cases, subcultures from positive presumptive tests upon the litmus lactose agar plate for the purpose of isolating and identifying the gas former fail to yield acid forming colonies. In these instances it is customary to interpret the result as non-significant so far as concerns pollution, since Creel (1914) has pointed out the rôle of sporulating lactose fermenting anaerobes in the presumptive test, a point which the latest standard methods seem not to have emphasized sufficiently. Even when Whipple (1903) used the glucose broth presumptive test following its original suggestion by Theobald Smith (1893) (1895) and Jackson (1906, 1907), modified this by substituting lactose bile, it was known, according to Winslow (1916), that certain organisms other than *B. coli* might be responsible for positive tests, but the factor of error introduced thereby was believed to be nearly negligible. Unfortunately Whipple's paper, which we quote from Professor Winslow's, is not available, but

it is clear that Jackson did not appreciate the importance of the anaerobes when he said, "no gas producer or mixture of gas producing bacteria will give results as high as 25 per cent gas except *B. coli*, even when three days' incubation is employed." Further experiences have shown the necessity of not relying simply upon the presumptive test as a criterion of pollution; Fuller (1915) found a proportion of positive presumptive tests in which *B. coli* could be confirmed ranging from only 60 to 90 per cent in 1 cc. samples, and Cumming (1916) found a percentage of *B. coli* to total gas formers ranging from 47.5 to 96.3. Moreover the amount of gas produced is no criterion, as is suggested, even in the most recent Standard Methods, since many of the sporulating anaerobes completely fill the closed arm of the fermentation tube with gas.

A recent experience by the Bureau of Sanitary Engineering of the State Board of Health of California (1916) at Sacramento is pertinent, where doses of chlorine so excessive as to cause disagreeable tastes and odors were being used by the local authorities to destroy *all* lactose fermenting bacteria in the water in the belief that this was necessary to insure its freedom from typhoid bacilli. It was indeed shown that the dosage of chlorine could be safely reduced from 2.6 pounds per 1,000,000 gallons to 1.3 pounds in fairly clear water (30 parts per million turbidity—for the dosage has to be varied according to turbidity), eliminating *B. coli* and presumably the typhoid bacillus, if present, without leaving any trace of free chlorine. With water so treated, however, presumptive tests were frequently positive, due to certain sporulating anaerobes from among which Mr. Frank Bachman succeeded in isolating *B. welchii*, a culture of which he has kindly given us.

SELECTIVE INHIBITION IN THE PRESUMPTIVE TEST

It is the purpose of this paper to describe a method to prevent gas formation by these anaerobic organisms, so that a greater proportion of positive tests is referable to coliform bacilli alone. This we have proposed to accomplish through

selective inhibition by gentian violet. Incidentally the Gram-positive sporulating aerobes, among which occasional forms capable of giving positive presumptive tests occur, are also inhibited.

The early literature dealing with the germicidal and antiseptic action of dyes has been sufficiently reviewed in a former publication (1914). Churchman's (1912) work, which was the point of departure for that paper, and which enabled him to state that a majority of Gram-positive organisms are inhibited in their growth by gentian violet, whereas most Gram-negative organisms grow well in its presence, dealt mostly with aerobic cultures. Certain anaerobes, e.g., two strains of *B. tetani*, were found by him to follow the rule as we have already amply confirmed (Hall and Taber, 1914). But others, namely *B. welchii* and *B. sporogenes*, were noted as exceptions to the general rule, i.e., while Gram positive, they were classed as resistant to the dye in the concentration used.

Inasmuch as we have undertaken a comparative study of a series of cultures of organisms belonging to the group of Gram-positive sporulating anaerobes, it seemed that it would be of interest to determine what, if any, difference could be found in their behavior and growth in the presence of gentian violet. As often happens, this study, which was undertaken purely from academic motives, was nearly completed before we appreciated its very pertinent practical utility.

THE ACTION OF GENTIAN VIOLET UPON CERTAIN GRAM POSITIVE SPORULATING ANAEROBES

The cultures of anaerobes tested came from various sources as indicated below. Most of them have been tentatively identified but we do not wish to emphasize the question of identity of any, except such as are designated "confirmed," since we have found certain atypical features in some of the cultures which may necessitate re-naming them. All are Gram-positive sporulating obligative anaerobic bacilli and, with the exception of *B. tetani* and *B. putrificus*, of clostridial morphology. The

cultures are free from aerobic contamination, but in some cases we feel certain two or more species of anaerobes are present in a single culture. For the purpose of this paper we have not yet undertaken to purify them—hence our hesitancy to emphasize the question of identity.

The first tests were made in deep agar according to the technic used by one of us (I. C. H.) with Mr. Taber (1914) in connection with the tetanus bacillus, but this proved not to be feasible in studying a larger number of cultures, owing to the care necessary in preparing and inoculating the tubes; it was replaced by the simpler method of tubing the media, after the desired concentration of dye had been secured by addition of the proper amount of a 1 per cent solution followed by sterilization in the Arnold sterilizer. In some of the earlier tests, crude glucose was used by oversight, resulting in decolorization which we attribute to residual SO_3 used as a bleaching agent in the preparation of the glucose and which is known to reduce dyes of the gentian violet group to their leuco-bases. A medium made with chemically pure glucose does not decolorize gentian violet. The reaction of the media is no doubt important, although stronger acids are required to decolorize gentian violet than is the case with alkalis; the reaction was adjusted to +1. Heavy seed cultures were prepared in the constricted tube described by Hall for the cultivation of anaerobes in glucose broth (1915) After twenty-four hours incubation at 37°C., 1 cc. was transferred to the tubes of melted glucose agar with the dye cooled to 40 to 45°C—rolled to mix thoroughly and incubated at 37°C. Control tests were always made in similar media without dye. Control tests for aerobic contamination were also made from each seed tube on agar plates in each experiment. Further, all positive growths were subcultured on agar slants or plates to detect possible aerobic contamination. All of the organisms produce gas even in the absence of fermentable carbohydrates; this fact and the appearance of definite colonies in the depths served as the criteria of growth. In all cases also, growth is accompanied by decolorization of the dye. The results of the tests are tabulated in table 1.

TABLE 1

Action of gentian violet upon certain Gram-positive sporulating anaerobes

CULTURE NUMBER	SOURCE	LABELLED	CONFIRMED	CONTROL NO DYE	GENTIAN VIOLET		
					1-100,000	1-10,000	1-1,000
51	Am. M. Nat. Hist.	B. botulinus no. 595	Yes	+	+	- - - - - -	- - - - - -
77	Cow dead of blackleg		B. chauvei	+	+	- - - - - -	- - - - - -
80	(*)	B. tetani	No—non-toxic	+	- +	- - - - - -	- - - - - -
82	Sterility test			+	- +	- - - - - -	- - - - - -
83	(*)	B. oedematis-Pasteur	No—probably B. chauvei	+	+	- - - - - -	- - - - - -
84	Dr. K. F. Meyer	B. chauvei-Munich	Yes	+	+	- - - - - -	- - - - - -
86	(*)	B. oedematis-Pasteur	Doubtful—contaminated?	+	+	- - - - - -	- - - - - -
89	Autopsy no. 1535. Gas infection			+	- +	- - - - - -	- - - - - -
105	(*)	B. tetani	No—non-toxic	+	+	- - - - - +	- - - - - -
113	Dr. K. F. Meyer	B. oedematis-"Koch"	Yes	+	+	+	- - - - - -
114	Dr. K. F. Meyer	Ghon-Sachs	Yes	+	+	- - - - - -	- - - - - -
115	Dr. K. F. Meyer	Vibrion Septique	Yes	+	+	- - - - - +	- - - - - -
133	Am. M. Nat. Hist.	B. welchii no. 521	Yes—but contaminated	+	+	+	- - - - - -
134	(*)	B. sporogenes 120	Doubtful	+	+	+	- - - - - -
136	(*)	B. tetani	No—non-toxic	+	+	- - - - - -	- - - - - -
139	Rabbit infection			+	- - - - - -	- - - - - -	- - - - - -
141	Rabbit infection			+	+	+	- - - - - -
165A	Dr. E. C. Dickson	B. botulinus	Yes	+	+	- - - - - +	- - - - - -
165B	Dr. E. C. Dickson	B. botulinus	Yes	+	+	- - - - - -	- - - - - -
165C	Dr. E. C. Dickson	B. botulinus	Yes	+	- +	- - - - - -	- - - - - -
178A	Canned corn		B. botulinus	+	+	- +	- - - - - -

189	Mr. Frank Bachman	B. welchii	Yes	+	+	+	– – – – –
191	Dr. W. S. Sturges	B. putrificus	Yes	– – +	– – +	– – – – –	– – – – –
192	Dr. W. S. Sturges	B. putrificus	Yes	– – +	– – +	– – – – –	– – – – –
193	Dr. W. S. Sturges	B. putrificus	Yes	– – +	– – +	– – – – +	– – – – –
198	Cutter Biological Laboratory	B. tetani	Yes	+	– +	– – – – –	– – – – –
210†	Water Sample—Table 3			+	+	– – – – –	– – – – –
215†	Sewage—table 5			+	+	– +	– – – – –
216†	Water Sample—table 5			+	+	– – – – –	– – – – –
230†	Soil			+	+	+	– – – – –
232	Dr. K. F. Meyer	Ghon-Sachs no. 1	Yes	+	– +	– – – – +	– – – – –
233	Dr. K. F. Meyer	B. chauvei-Pasteur	Yes	+	– – – – –	– – – – –	– – – – –
234	Dr. K. F. Meyer	B. chauvei-Cal.	Yes	+	+	– – +	– – – – –

* Withheld by request.

† Included in last test only.

– = no growth; + = growth

Daily observations up to and including five days, indicated by repeated marks.

None of the cultures grew in glucose agar containing 1–1,000 gentian violet and only 14 out of 33 showed growth in a concentration of 1–10,000. Of these latter 6 grew within one day, 2 within two days, 1 in three days and 5 only within five days. In all of these there was marked evidence of restriction by the dye. In a concentration of 1–100,000 all the cultures but 2 grew—mostly quite vigorously, with gas formation and marked decolorization of the dye. All the controls grew vigorously except 3 cultures of *B. putrificus* which, not fermenting glucose, are characterized by somewhat more sluggish development. The tests were repeated with most of the cultures three times with essentially similar results.

In spite of the fact that according to the above tests more than 1 part gentian violet in 10,000 of agar is required to completely inhibit the growth of these organisms, we conceived that a smaller amount might suffice to inhibit spurious presumptive tests in lactose broth due to organisms of this group. There are two reasons supporting such a supposition, first, the conditions of anaerobiosis in the Durham fermentation tube are not so favorable, second, it is believed that the colloidal nature of the agar reduces the efficiency of the dye, thereby making a higher concentration necessary to accomplish a given result.

A test of these organisms was therefore made in Durham fermentation tubes with 1–100,000 gentian violet in 1 per cent lactose broth. The test was controlled by means of a series of tests without the dye, all of which developed growth excepting cultures 115 and 192; their failure is attributed to the fact that in these tests no special precautions were taken to eliminate oxygen. All but six of the control tests produced gas in addition to turbidity. Of the dye tests however only one produced either gas or turbidity and this was shown to be aerobically contaminated. It may be mentioned that the somewhat inferior anaerobiosis of the Durham tube for pure culture of anaerobes is compensated in routine presumptive tests for *B. coli* in water by the practically constant presence of aerophilic organisms which reduce the oxygen tension sufficiently to provide suitable conditions of anaerobiosis. We may admit also that the writer's

modification (1914) of the Durham fermentation tube is less apt to provide anaerobic conditions than the usual type.

In order to test the applicability of gentian violet in actual water examinations, arrangement was made to secure samples showing positive presumptive tests from the Bureau of Sanitary Engineering of the State Board of Health of California. Our sincere thanks are due Messrs. C. G. Gillespie and Frank Bachman for their courtesy in this matter. Inasmuch as the samples were frequently several days old before we received them it is not surprising that our findings do not coincide exactly.

TECHNIC

Upon receipt the bottles were thoroughly shaken and the water divided into two parts; one portion was heated to 56°–60°C for thirty minutes, the other being left unheated. Each of these was again subdivided into two parts, one of which was inoculated into a Durham fermentation tube containing 2 per cent lactose broth with gentian violet, the other being inoculated into a tube of similar medium without gentian violet. The tubes contained 10 cc. of double strength media and the amount of water inoculated was 10 cc. in each case. The final concentration of lactose was therefore 1 per cent; the final concentration of dye was 1–100,000 in the first 21 samples, after that 1–20,000. Tests at the latter concentration were also made with what remained of the first 21 samples. The change was made because gas was encountered several times in the unheated sample inoculated into dye broth from which no aerobic gas former could be isolated; we interpreted this result as due to imperfect inhibition of gas forming anaerobes, indeed we had anticipated that *B. coli* should be isolated from every unheated sample showing gas in the presence of 1–100,000 gentian violet. As later shown even 1–20,000 is not sufficient to give this result, due, we believe, to the somewhat better conditions of anaerobiosis provided by the presence of aerobic organisms.

Incubation was at 37° until gas formation occurred, if within five days. Plates of litmus lactose agar were then streaked

and acid-forming colonies isolated upon the unused half of the plate. After all but 22 samples had been examined we began to realize that there are possibly two conditions in which the positive presumptive test may truly be due to *B. coli* and yet great difficulty be encountered in the isolation of the proper organisms due to the fact that no red colonies appear on the litmus lactose agar plate.

The first of these is the occurrence of so-called "attenuated" coliform bacilli which may be considered to have temporarily or partially lost their property of fermenting lactose under aerobic conditions. Many water analysts are well aware of this occurrence and so the new standard methods for 1917 emphasize the necessity of testing suspicious looking surface colonies of true morphology in lactose broth for gas formation, even though they do not produce acid upon the litmus lactose agar plate. But it is to a degree only accidental that this procedure sometimes results in identification of *B. coli;* in our experience blue surface colonies usually, but not invariably, fail to form gas in the fermentation tube. Our work holds no suggestion as to the elimination of this difficulty.

The second condition is that in which strongly proteolytic organisms, e.g., certain of the hay bacillus group, not fermenting lactose, liberate sufficient alkali to neutralize any acid due to *B. coli* in the plates, thus suppressing the appearance of red colonies except when widely separated; a further aggravation is that these organisms sometimes have marked spreading proclivities. Fortunately the use of gentian violet is quite efficacious in preventing this particular source of trouble, since most aerobic sporulating bacteria are inhibited as well as the Gram-positive sporulating anaerobes. It was quickly determined that the addition of 1–100,000 gentian violet to litmus lactose agar served admirably to inhibit the undesirable organisms, not always completely, but at any rate sufficiently to prevent their spreading and interfering with the display of acid by *B. coli.* The color imparted by this amount of dye is quite insufficient to mask the desired color changes in the litmus. In advocating the use of gentian violet in this manner for the

isolation of *B. coli* we appreciate of course that the method is essentially the same as that used by Drigalski and Conradi (1902) in the isolation of *B. typhosus*, only the aim is slightly different.

Isolated coliform colonies were stained by Gram's method as modified by Stovall and Nichols (1916) and if typical Gram negative coccobacilli were present, they were tested in lactose broth for gas formation and in gelatin for non-liquefaction. These confirmatory tests were made at 37° C. for forty-eight hours, gelatin being tested for solidification by immersion in ice-water. In case only non-acidifying colonies appeared, at least two were similarly isolated and if otherwise coliform were tested in lactose broth to detect so-called "attenuated" *B. coli*, as required by the 1917 Standard Methods for the Examination of Water and Sewage.

Since our hypothesis involved the demonstration of *B. coli* in as many cases as possible where gas was produced in the presence of gentian violet, we adopted a uniform procedure of repeating the above tests with a subculture into a new tube of lactose broth from the original presumptive, if the first plate failed to show typical coliform acidifiers, or if the identification of *B. coli* failed, in either of the tests of the unheated sample.

The tabulation of the complete data occupies so much space that it seems best only to summarize the findings for the sake of economy, as in table 2.

Unquestionably the most interesting feature in table 2 is the behavior of the heated samples. In only one case was a positive presumptive test secured in the broth containing the dye as against 18 positive tests without it; in fact practically all of the tubes with dye appeared to be sterile. Aerobic spores as well as anaerobic spores were inhibited. The single tube mentioned showed slight gas only after four days' inoculation and this was found to be due, neither to *B. coli* nor necessarily to anaerobes; a lactolytic acid and gas forming sporulating bacillus was secured from the plates. This culture in fact was the starting point of our use of gentian violet in the lactose agar plate inasmuch as repeated tests from the broth failed to show

the slightest growth upon such a plate. The same organism was isolated from the presumptive test without gentian violet, and we believe its presence in the unheated sample was the reason for our failure to secure *B. coli* from this sample, from the presumptive test either with or without gentian violet although on the occasion of two other independent tests *B. coli* was isolated from the same sample. At least 16, or 76 per cent, of the 21 samples contained gas-forming anaerobes, as shown by positive presumptive tests in plain lactose broth with the heated samples from which only non-lactolytic aerobes appeared on the litmus-lactose-agar plate subcultures; these were stained by

TABLE 2

Tests for B. coli and sporulating gas-forming anaerobes in 21 samples of water—using 1–100000 gentian violet in the presumptive test

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH		SAMPLES SHOWING ACID COLONIES ON LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
				First trial	Second trial	Total
Not heated	1–100,000 gentian violet,	20	14	11	4	15
	No gentian violet,	21	12	8	4	12
Heated 56°–60°C.; 30 minutes	1–100,000 gentian violet,	1	1*	0	0	0
	No gentian violet,	18	2*	0	0	0

* Gas in presumptive tests and acid on plates due to lactolytic aerobic spore bearers.

Gram's method and were found to be Gram-positive sporulating bacilli with one exception which contained Gram-positive cocci. Two heated samples also giving positive presumptive tests in plain lactose broth yielded acid colonies on litmus lactose agar; the organisms were lactolytic aerobic spores. Anaerobic spores may also have been present but we cannot be sure, and the percentage of samples certainly having anaerobic spores is given exclusive of these two.

As table 2 shows, all the samples showed gas in the plain lactose broth tube inoculated with 10 cc. unheated water, as against only 20 in the gentian violet lactose broth. But of the 20 there were 14 which produced acid colonies on litmus-lactose-

agar, as against 12 out of the 21. And of the plates from the gentian violet presumptive tests, 15 samples yielded *B. coli* whereas only 12 of the samples tested on plain lactose broth yielded *B. coli.* The discrepancy in favor of gentian violet in the presumptive test is still further shown in the greater proportion of cultures secured in the first test, 73 per cent from the dye series, as against 67 per cent from the non-dye presumptive. Strangely enough, the odd sample failing to show a positive presumptive test in the presence of gentian violet yielded *B. coli* from the non-dye tube. Looking ahead to the data formulated in table 3 in which gentian violet 1–20,000 was used we find that this particular sample yielded *B. coli* from the test, with, as well

TABLE 3

Tests for B. coli and sporulating gas-forming anaerobes in 20 samples of water—using 1–20,000 gentian violet in the presumptive test

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH		SAMPLES SHOWING ACID COLONIES ON LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
				First trial	Second trial	Total
Unheated..........	1–20,000 gentian violet,	15	5	4	8	12
	No gentian violet,	20	7	5	9	13
Heated 56°–60°C.; 30 minutes.......	1–20,000 gentian violet,	0	0	0	0	0
	No gentian violet,	19	1	0	1	1

as without, the dye. We cannot explain this anomaly. Our failure to isolate *B. coli* from five unheated samples showing gas in the presumptive test led us to increase the concentration of dye to 1–20,000. The unused residues of 20 of the first 21 samples were therefore re-examined with this change in technic along with new samples secured. Table 3 summarizes the results of this second series of tests with these 20 samples. Part of the differences in findings are no doubt due to the greater age of the samples when the last tests were made.

This time as in the first instance the selective inhibition of sporulating gas formers is shown in the case of the heated sample tested in lactose broth containing gentian violet; not one showed

any trace of gas in five days. Inasmuch as only one of the 19 heated samples showing gas in the test without the dye produced acid on the lactose agar plate, due incidentally to *B. coli* which was isolated and must be considered to have escaped the heating, we are justified in the statement that at least 90 per cent of these samples contained gas forming sporebearing anaerobes, and considering the two series of tests, we can say that only one of the whole 20 samples failed both times to show gas necessarily interpreted as due to anaerobic sporebearing bacilli; in other words 95 per cent of these particular samples contained organisms in addition to *B. coli*, capable of giving rise to a positive presumptive test in lactose broth without gentian violet. The organisms growing upon the litmus lactose agar plates, though non-acidifying, barring the one sample in which *B. coli* was found, were stained by Gram's method and were found in every case to be Gram-positive sporulating bacilli of the hay bacillus group.

All the unheated samples showed gas in the standard presumptive test as against 15 in the gentian violet lactose broth. The proportion showing acid colonies from each set was approximately one-third—considerably lower than in the first series, owing to the disappearance of *B. coli* from some of the samples on standing. Notwithstanding the low proportion of samples showing acid colonies, we were able to isolate *B. coli* from 13 of the 15 showing gas in dye broth and from 14 of the 20 showing gas in plain lactose broth. One sample showing *B. coli* in the standard test failed to show it in the dye test. From the other sample showing gas in gentian violet lactose broth a culture tentatively designated as *B. cloacae* was isolated. This organism, as usually described, differs from *B. coli* only in possessing the ability to liquefy gelatin. We have had a number of such cultures which will be mentioned in the further discussion. In each case we have attempted, and usually successfully, to separate such cultures into subcultures, one of which liquefies gelatin and does not ferment lactose, the other of which ferments lactose and does not liquefy gelatin; in other words, *B. coli*. The principal organisms thus separated from *B. coli* have

been identified as *B. proteus* or *B. fluorescens-liquefaciens.* In all cases the original colonies were well separated on the plate, and usually we have been at a loss to account for the association of two separable species in a supposedly pure culture giving the reactions ascribed to *B. cloacae.* In some other cases contaminations subsequent to the primary isolation were responsible; this was assumed in those few instances when the gelatin liquefier proved to be a spore bearer. At any rate we have come to question whether so-called *B. cloacae* is not often a mixture of a gelatin liquefying non-lactose fermenter with *B. coli.* In our studies all such organisms were planted on plain agar and set aside for further study. Unfortunately in some of them we lost one of the organisms, or must we say, the culture lost its lactolytic *properties?* In the case first referred to above the culture would not ferment lactose when re-tested, though it had formerly done so actively; it conformed therefore to the usual description of *B. proteus* and we conceive that we lost *B. coli.* In one other case of this series we clearly separated *B. proteus* from *B. coli* by plating out in gelatin from a fermentation tube of lactose broth inoculated with what appeared to be *B. cloacae.*

An interesting point in this second series is that the proportion between 1st and 2nd trials at isolation of *B. coli* is reversed, as compared to the 1st series, over one-half of the successful isolations coming from the second trial, indicating, we believe, a loss in number and vigor of *B. coli* due to storage. There were three samples in which *B. coli* was found the first time but not the second; the smallest amounts of water in which *B. coli* could originally be found, according to the Bureau of Sanitary Engineering were respectively 10 cc., 1 cc., and 0.1 cc. There were two samples originally containing *B. coli* in 10 cc. and 1 cc. respectively in which *B. coli* was found the second time and not the first and one sample originally showing *B. coli* in 10 cc. in which we never found it. As against these data showing the disappearance of *B. coli* from standing samples of polluted water we have only one case in which the presence of gas forming anaerobes could not be corroborated in the repeated test.

Since 5 samples, unheated and tested in gentian violet lactose broth failed to produce gas in five days, we conclude that *B. coli*, present as shown by previous tests, had disappeared from the sample. All 5 showed gas in the presumptive test without gentian violet—interpreted in all but one, from which *B. coli* was isolated with some difficulty—as due to anaerobes.

In addition to the last tests, examinations were made of 22 fresh samples, according to the same technic. The data of table 4 are comparable, therefore, to those of table 3 except that the samples had not stood so long.

TABLE 4

Tests for B. coli and sporulating gas-forming anaerobes in 22 samples of water—using 1–20,000 gentian violet in the presumptive test

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH		SAMPLES SHOWING ACID COLONIES ON LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
				First trial	Second trial	Total
Unheated	1–20,000 gentian violet,	18	14	14	1*	15
	No gentian violet,	22	12	12	1*	13
Heated 56°–60°C.; 30 minutes	1–20,000 gentian violet,	0	0	0	0	0
	No gentian violet,	22	1†	1†	0	1

* Same sample—"attenuated" B. coli fermenting lactose anaerobically but not aerobically.

† Same sample—B. coli not killed by heating.

Analysis of the data shows that this series of samples possessed anaerobic sporulating gas formers in at least 19, or 95 per cent, of the samples according to the standard presumptive test in lactose broth with the heated specimens. In the odd case *B. coli* which had escaped the heating was isolated; its occurrence in the standard test, and not in the dye test of the heated sample, must have been fortuitous since the organisms could be shown not to be inhibited by gentian violet. There were no positive presumptive tests with the heated sample having gentian violet. Of the positives in the standard test 12 were tested on a lactose agar plate containing 1–10,000 gentian violet; in only one case did growth appear, which consisted of a mixture of thick Gram

positive and thin Gram negative sporulating rods. An attempt at repeated demonstration of the non resistance of this particular culture to gentian violet failed, but this and other observations show that exceptional strains of hay bacillus may be found which are not inhibited completely by gentian violet.

In the unheated samples there were 11 in which *B. coli* was isolated from both the dye and the non-dye tests. In the case of one of these in gentian violet lactose broth 2 cultures, apparently *B. cloacae,* were isolated from well separated acid colonies on litmus lactose agar. One of these was subsequently separated into 2 subcultures having the properties of *B. fluorescens-liquefaciens* and *B. coli* respectively; the other after a delayed period was found to have lost the property of *B. coli*, i.e., lactose fermentation, formerly possessed, and retained only the characteristics of *B. fluorescens-liquefaciens.* Our interpretation of this phenomenon has already been discussed.

Four unheated samples yielded *B. coli* from the dye test and not from the standard test. In 2 of these the plates were clearly overgrown by spores which we feel so masked the colonies and acid produced by *B. coli* that we could not pick out the colonies; in the other 2 no acid was displayed in either the first or second tests, and the picked colonies proved not to be the colon bacillus.

Two unheated samples yielded *B. coli* from the standard test and not from the dye test. In one of them the presumptive test was positive only after three days, and the non-acid colonies picked from the plate failed to ferment lactose; the second presumptive test was negative for five days. In the other case a culture tentatively called *B. cloacae* was isolated; subsequently examined with a view to the separation of the unheated *B. coli* from the gelain liquefier, it was found that the culture presented only the characteristics of *B. proteus.*

Five unheated samples failed to yield *B. coli* from either the standard or the dye test. In 4 of these the presumptive test with dye was negative during five day's incubation; the fifth showed a bare trace of gas on the fifth day only. In the standard test all showed gas, 1 on the second day, 2 on the third, 1 on

the fourth and 1 on the fifth. In no case were acid colonies developed on the plates. All were due to sporulating anaerobes as shown by the corresponding tests with the heated fractions of these samples. All these samples had originally contained *B. coli* according to the Bureau of Sanitary Engineering though in no case in less than 10 cc.

At this stage in the investigation we were ready to adopt the use of 1–100,000 gentian violet in all of the litmus lactose agar plates as a routine procedure in the isolation of *B. coli* from positive presumptive tests, upon the basis of experimental trials with pure cultures of *B. coli* and the hay bacillus. The

TABLE 5

Tests for B. coli and sporulating gas-forming anaerobes in 22 samples of water—using 1–20,000 gentian violet in the presumptive test and 1–100,000 gentian violet in the litmus lactose agar plates

TREATMENT OF SAMPLE	SAMPLE SHOWING GAS IN LACTOSE BROTH	SAMPLES SHOWING ACID COLONIES ON GENTIAN VIOLET LITMUS LACTOSE AGAR	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED		
			First trial	Second trial	Total
Unheated	1–20,000 gentian violet, 21	15	15	4	19
	No gentian, violet, 22	14	14	6	20
Heated 56°–60°C.; 30 minutes	1–20,000 gentian violet, 4	2	1*	0	1
	No gentian violet, 22	4	1*	0	1

* Not the same sample.

considerations involved in this change of method have already been discussed fully under the heading of "technic." Twenty-two new samples were tested according to this plan as shown in table 5.

At least 18, or over 81 per cent, of these samples contained anaerobic sporulating gas formers, as judged by the positive tests in the standard presumptive inoculated with heated samples. In 1 case *B. coli* escaped the heating and was isolated and identified as the gas former; in 3 others, Gram-positive-aerobic sporulating gas formers belonging to the hay bacillus group were isolated. The recognition of members of this group

as capable of giving rise to a positive presumptive test in lactose broth is important for these organisms are usually non-lactolytic. Peculiarly these organisms grew, though slowly, upon the gentian violet litmus lactose agar used for plating; their colonies could easily be differentiated from the colon bacillus by the marked manner in which they absorb the dye from the media. In only 1 of the 3 was there a suggestion of acid reaction in the plate and this was anything but marked. It suggests that possibly these organisms produce gas through putrefaction, as most of the anaerobic spore bearers are known to do in the absence of fermentable carbohydrates. It also raises the question as to whether some of the spurious presumptive tests which bacteriologists have been in the habit of ascribing to anaerobic spores may not be due to aerobic spores. This brings us to another interesting point, namely that 16 of the gentian violet litmus lactose agar plates made from these standard presumptive tests with heated water samples not only showed no acid, but were absolutely barren for at least forty-eight hours. It will be remembered that these plates in some of the other series showed mainly, if not entirely, Gram-positive bacilli of the hay bacillus group. The inhibition of this group in this last series of tests is no doubt responsible for the fact that in the two other instances plates showing acid colonies due to streptococci were recorded. We recall that Krumwiede and Pratt (1914) found streptococci and pneumococci somewhat more resistant to gentian violet than some other Gram-positive bacteria.

None of these heated samples however gave positive presumptive tests in the lactose broth with gentian violet. The four positives obtained were from samples of which plates from the standard test were sterile, as mentioned. In one case the fermentation tube was full of gas in twenty-four hours; *B. coli* was isolated from among the acid colonies on the plate. In one other an aerobic, acid forming spore was found to comprise the single colony on the plate. The presumptive test in this instance though showing no gas till the fourth day then developed a tube full, at the same time showing a marked decolorization of the dye. The 2 other positive presumptives showed gas only

after three and four days respectively and failed to give aerobic growth on lactose or plain agar with or without gentian violet; they were found to contain apparently pure cultures of sporulating anaerobes. There has not been time to identify these organisms with certainty, but finding them in the positive presumptive test with gentian violet indicates that for the best results we shall have to increase the concentration of dye somewhat over that with which we have hitherto experimented, although even a concentration of 1–20,000 gives nearly perfect results, as we have shown. A comparison of these cultures, numbers 210, 215 and 216 as to their resistance to gentian violet, in table 1, has shown that they are not unique in this respect.

Among the unheated samples *B. coli* was isolated from both the standard and dye presumptive tests eighteen times. In one sample tested in dye broth another instance of supposed *B. cloacae* turned out to be a mixture of *B. coli* and *B. proteus*.

One sample yielded "attenuated" *B. coli* with difficulty from the dye test and not from the plain lactose broth test. It is safe to say that the organisms could not have been isolated from plain litmus lactose agar without the dye, since the culture in the non-dye tube was heavily overgrown with a member of the hay bacillus group, which did not appear on the plates, however.

Two samples yielded *B. coli* from the standard test and not from the dye test; the gentian violet litmus lactose agar plates made from the last failed on two successive occasions to show aerobic growth. Unfortunately the gas formers, probably dye resistant anaerobes, (though these samples were not the same as those from which we recovered the pure cultures of anaerobes in the heated fractions) were lost before the next subculture was successfully made. We were surprised to find that these last samples were originally heavily contaminated, yielding *B. coli* in 0.001 cc., according to the record of the Bureau of Sanitary Engineering. The result seems to support the idea expressed by Mr. Frank Bachman (1917) that it is such samples from which *B. coli* disappear most readily on standing.

One sample failed to yield *B. coli* in any of our tests; anaerobes were present however, giving positive presumptive tests

in the standard broth, but totally negative in the presence of gentian violet. According to the Bureau of Sanitary Engineering *B. coli* was originally isolated in 20 cc. only and was therefore considered to have died out from the sample.

Again we can call attention to the disproportion between 1st and 2nd successful isolations of *B. coli* favoring the use of gentian violet in the presumptive test, although in this series of tests the total number of samples yielding *B. coli* in the dye test was exceeded by one in the standard test, due to the fortuitous circumstances above mentioned.

TIME OF FIRST APPEARANCE OF GAS

Standard methods permit a negative report on presumptive tests failing to show gas in forty-eight hours. In the beginning of our work we adopted a somewhat longer period, namely five days, in order to isolate as large a proportion as possible of colon bacilli present. It is interesting to note that in no case was *B. coli* isolated from a presumptive test first showing gas after four days. With gentian violet 77 per cent of the tests from which *B. coli* was isolated showed gas within forty-eight hours, without gentian violet, 88 per cent. The figures are not quite representative, however, as we find on analysis that the presence of gentian violet in the presumptive test does not generally delay appearance of gas. The somewhat unfavorable percentage is due almost entirely to a small group of samples tabulated in table 5. These were originally very badly contaminated, but had almost eliminated *B. coli* on standing; if table 5 were excluded the result would stand 88 per cent positive within forty-eight hours with gentian violet, to 87 per cent without. The data for all the tests are tabulated in table 6. The main point seems to be that we are justified in waiting at least four days before calling the presumptive test negative.

The positive tests from which it was impossible for us to isolate *B. coli* are also tabulated, in table 7.

The figures include 1st trials many of which failed to yield *B. coli* but from the subcultures of which in a second presump-

tive test a successful isolation was made. The highest total number of positive tests in this series, from which *B. coli* could not be isolated, consists of course of tests made of heated samples in the standard lactose broth. We feel justified in interpreting these as due mainly to sporulating anaerobes although the figures contain those few cases in which lactolytic aerobic spores were isolated, as do also the few cases showing a test in the presence of the dye. The general tendency of tests not showing *B. coli* is delayed gas production; taking all the tests in table 7 more than 32 per cent occurred after the second day.

A general conclusion from tables 6 and 7 is that the time of appearance of gas gives no certain clue to the nature of the gas former.

AMOUNT OF GAS

Any arbitrary amount of gas required in the presumptive test to be interpreted as a positive test is apt to be misleading, because the presumptive test frequently deals with mixed cultures, and we believe usually containing other gas formers aside from *B. coli*. And by no means most of these produce less than 10 per cent gas in the closed arms; on the other hand many cultures which we can not consider other than members of the colon group produce less than 10 per cent gas and indeed certain coliform bacilli ferment lactose with the formation of acid only. While none of the latter has been so designated in this study because we are concerned with the question of gas formers in the presumptive test, yet the writers are strongly inclined toward Kligler's (1914) view that acid formation is a true criterion of fermentation by *B. coli*. Levene (1916), on the contrary, has held that gas formation is the true evidence of fermentation. Frequently, as Levene suggests, no doubt usually, in the case of *B. coli*, the two phenomena are correlated. In the case of many anaerobes, however, and possibly of some aerobic organisms, gas is produced abundantly even in the absence of fermentable carbohydrates and it is just this which has made the former attempts at classification of the anaerobic spore bearers on the basis of gas formation so confusing.

Because of our skepticism as to the importance of the *quantity* of gas in the presumptive test, complete records were not kept. Since the appearance of the new standard methods, however, we have made numerous observations which need not be tabulated to show that the amount of gas appearing in the presumptive

TABLE 6

First appearance of gas in presumptive tests from which B. coli was isolated

TREATMENT OF SAMPLE	LACTOSE BROTH	DAYS					
		1	2	3	4	5	Total
Not heated.....	Gentian violet	30	17	4	10	0	61
	No gentian violet	35	18	3	2	0	58
Heated.........	Gentian violet	1	0	0	0	0	1
	No gentian violet	1	0	0	2	0	3
Total............................		67	35	7	14		123

TABLE 7

First appearance of gas in presumptive tests from which B. coli was not isolated

TREATMENT OF SAMPLE	LACTOSE BROTH	DAYS					
		1	2	3	4	5	Total
Not heated.....	Gentian violet	13	10	5	2	3	33
	No gentian violet	29	16	7	4	3	58
Heated.........	Gentian violet	0	0	2	3	0	5
	No gentian violet	21	31	10	14	3	79
Total............................		63	57	24	22	9	175

test is of little importance. *B. coli* may frequently be isolated from tubes showing only a trace of gas or tubes full of gas may be due to sporulating aerobes or anaerobes only.

SUMMARY

This report deals with the question of the true interpretation to be placed upon the presumptive test for *B. coli* as a criterion of polluted water. It is pointed out that spurious tests are

frequently due to sporulating organisms of little or no significance from the sanitary viewpoint. These are mainly anaerobic bacilli, but aerobic gas producing spore bearers also occur. Both groups are inhibited by gentian violet in proportion to the concentration, and, whereas *B. coli* is not inhibited by a much greater concentration of this dye, its use for the selective inhibition of the former organism is urged. Tests were made at a concentration of 1–100,000 and 1–20,000 in the presumptive test and at 1–100,000 in a litmus lactose agar plating medium. In all, 65 samples of water, from which *B. coli* had previously been isolated, were tested; 54 were found to contain both sporulating anaerobes and *B. coli*, 1 contained only *B. coli* and no anaerobes, and 10 showed anaerobes only, *B. coli* having disappeared therefrom on standing. The tests showed that gentian violet in the presumptive test not only does not interfere with the isolation of *B. coli* but actually favors it according to the statistical analysis of the results. In the 10 samples set down as containing only anaerobes, presumptive tests were negative in the dye test—positive in the standard test. A most striking feature is the nearly complete inhibition of growth in the case of heated samples tested in dye broth—these containing, with a few exceptions, only sporulating organisms.

Some positive tests were obtained in gentian violet lactose broth from which *B. coli* could not be isolated; barring the possibility that these were due to anaerobic Gram-negative non-sporulating bacilli, it is believed they can be avoided completely by increasing the concentration of the dye. It may be suggested, however, that if one part gentian violet in twenty liters of lactose broth inhibits nearly 95 per cent of the spurious presumptive tests, as inspection of the proportion (4 to 78) of false tests obtained with and without gentian violet in the heated samples shows, it would scarcely be worth while to increase the concentration greatly for the extra 5 or 6 per cent. Even if we analyze the figures in the least favorable manner, i.e., to show the percentage of samples yielding *B. coli* from positive presumptive tests in all our examinations we have the following:

	POSITIVE PRESUMPTIVES	B. COLI ISOLATED	PERCENTAGE
Unheated samples:			
Tested in gentian violet lactose broth.........	74	61	82.4
Tested in standard lactose broth..............	85	58	68.2
Heated samples:			
Tested in gentian violet lactose broth	5	1	20
Tested in standard lactose broth.............	81	3	3.7

Thus while we cannot claim to have progressed to a point where it is safe to dispense with the litmus lactose agar plate and rely wholly upon the presumptive test, we have in the use of gentian violet a method which offers promise of even such a result in higher concentrations than we have used and in the lower concentrations provides a method of eliminating the great majority of spurious tests, obviating the necessity of much futile plating with a consequent saving in time and material.

REFERENCES.

Amer. Public Health Association 1917 Standard methods for the examination of water and sewage. 3d edition.

Bachman, Frank 1917 Personal communication.

Churchman 1912 The selective bactericidal action of gentian violet. J. Exp. Med., **16**, 221.

Clark and Lubs 1915 The differentiation of bacteria of the Colon-aerogenes family by the use of indicators. J. Infect. Dis., **17**, 160.

Creel 1914 Examination of drinking water on railroad trains. Hyg. Lab. Bull. no. 100.

Cumming 1916 Investigation of the pollution and sanitary conditions of the Potomac water shed. Hyg. Lab. Bull. no. 104.

Drigalski and Conradi 1902 Ueber ein Verfahren zum Nachweis der Typhusbacillen. Ztschr. f. Hyg., **39**, 283.

Fuller 1915 Biochemical and Engineering Aspects of Sanitary Water Supply. Jour. Franklin Inst., Ch. 30, 17.

Gillespie 1916 Report of the Bureau of Sanitary Engineering for November 1916. Cal. State Board of Health Bull. December 1916, **12**, 353.

Hall 1914 An improved (Durham) fermentation tube. Am. J. Pub. Health, **4**, 1173.

1915 A new aerobic-anaerobic culture tube. Univ. of Calif. Pub. in Pathology, **2**, 147.

Hall and Taber 1914 The effect of gentian violet on the *Bacillus tetani*, tetanus toxin and certain laboratory animals. J. Infect. Dis. **15**, 566.

JACKSON 1906–07. A new solution for the presumptive test for *Bacillus coli*. Biological studies by the pupils of W. T. Sedgwick. Univ. of Chicago Press, 1906.
The use of lactose bile medium in water. J. Infect. Dis., Supp. No. 3, p. 30, 1907.

KLIGLER 1914 Studies on the classification of the colon group. J. Infect. Dis. **15**, 187.

KRUMWIEDE AND PRATT 1914 Observations on the growth of bacteria on media containing various aniline dyes. J. Exp. Med., **19**, 20.

LEVENE 1916 Acid production and other characters of B. coli-like bacteria from feces and sewage. J. Infect. Dis., **19**, 773.

ROGERS, CLARK AND DAVIS 1914 The colon group of bacteria. J. Infect. Dis. **14**, 411.

ROGERS, CLARK AND EVANS 1915 The characteristics of the bacteria of the colon type found in bovine feces. J. Infect. Dis. **15**, 99.
The characteristics of the colon type occurring on grains. J. Infect. Dis., **17**, 137.

SMITH 1893 A new method for determining quantitatively the pollution of water by fecal bacteria. 13th Annual Report State Board of Health of New York, p. 712.
1893 The fermentation tube. Wilder Quarter Century Book, p. 187.
1895 Ueber den Nachweis des Bacillus Coli Communis im Wasser. Centralbl. f. Bakt. Abt. 1. orig. **18**, 494.

STOVALL AND NICHOLS 1916 Stabilized gentian violet. J. Am. Med. Assoc. **46**, 1620.

WHIPPLE 1903 Quoted by Winslow from The Tech. Quart. **16**, 18.

WINSLOW 1916 Tests for B. coli as an indicator of water pollution. J. Am. Water Works Assoc. **3**, 927.

A BRIEF NOTE ON THE USE OF GENTIAN VIOLET IN PRESUMPTIVE TESTS FOR B. COLI IN MILK WITH REFERENCE TO SPORULATING ANAEROBES

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In the case of milk, as formerly in the case of water, qualitative bacteriological standards have been preceded by quantitative methods of evaluation, a trend of evolution just opposite to that usual in science. The main reliance has been upon the plain agar plate count though this is now professedly inferior to the lactose agar plate for approximating the total aerobic plate count as recently shown by Sherman (1916). But these plating methods take no cognizance of anaerobes and other bacteria whose particular metabolic requirements happen not to be met by the conditions set, and so the most recent Standard Methods for Bacteriological Analysis of Milk (A. P. H. A. 1916) tend to lay emphasis upon the method of direct microscopic examination, the literature of which has been so adequately reviewed and the technic perfected by Breed and Brew (1916).

While appreciating the fact that the bacterial flora of milk is well known and that qualitative examinations are frequently made when searching for the cause of specific abnormalities in milk we note that such examinations have not as yet become a part of our routine procedure.

In California, however, the recent passage of laws (Cal. 1915) effective October 1, 1916, prohibiting the sale without pasteurization of milk from cows not shown to be free from tuberculosis by the tuberculin test, has suggested the presumptive test for *B. coli* as a criterion of unsuccessful operation of the pasteurizing apparatus, the presence of this organism being

assumed even in milk passably clean. Ayers (1917) has recently pointed out the significance of *large* numbers of *B. coli* in raw milk as indicating temperature conditions suitable for development rather than heavy initial contamination and his viewpoint is confirmed in its essentials by Harding's condemnation (1917) of the bacterial count as an index of cleanliness.

In attempting the use of the presumptive test for *B. coli* as a criterion of unsuccessful pasteurization, Mr. W. H. Stabler, a student in the Department of Veterinary Science, to whom we are indebted for the suggestion, found in a number of cases a situation apparently analogous to that often described in water, namely, gas formation in lactose broth tubes from which no *B. coli* could be isolated. The result was interpreted as due to sporulating anaerobes resistant to the temperature of pasteurization.

Accordingly, after testing the use of gentian violet in presumptive tests for *B. coli* in water (1918), we began testing milk samples under similar conditions, except that 1 cc. of milk was tested in each case, whereas the water tests were made with 10 cc. each. The amount of broth in each tube was 20 cc.

Plates of litmus lactose agar were streaked from tubes showing gas for isolation of *B. coli*. After the first six samples 1 part gentian violet in 100,000 was added to the litmus lactose agar to inhibit the growth of Gram positive spores (most of them non lactose fermenters), through whose spreading proclivities, combined with rather marked proteolytic activities, the acid produced by *B. coli* may be masked, as noted in the case of plates from the presumptive tests of water. A further advantage in the case of milk is that lactolytic cocci, which are notably frequent, are also inhibited, thus further favoring the liklihood of isolated acidifiers proving to be colon bacilli, though there is not much danger of confusing them because of the difference in type of colony. Further details of technic were the same as in the water tests.

The data obtained are summarized in table 1.

Thirty-three samples from ten different sources were tested, 13 of raw milk from tuberculin tested cows, 14 of pasteurized

milk, 2 of certified raw milk, and 4 not labelled. We do not wish at this time to lay great emphasis upon the findings of *B. coli* as distributed among these classes further than to say that a surprisingly large number of pasteurized samples, namely, 10 out of 14, contained *B. coli*.

What we do wish to emphasize is, (1), that no evidence was gained of the survival of anaerobes after heating to 56–60° for thirty minutes as shown by the presumptive test in lactose broth, with or without gentian violet; (2), that the use of gentian violet does not prevent the isolation of *B. coli* from unheated samples. Indeed 3 samples gave positive presumptive tests

TABLE 1

TREATMENT OF SAMPLE	SAMPLES SHOWING GAS IN LACTOSE BROTH	SAMPLES SHOWING ACID COLONIES IN LACTOSE AGAR		SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED
		No gentian violet	1–100,000 gentian violet	
Not heated.......	1–20,000 gentian violet, 23	5		2
			18	18
	No gentian violet, 20	3		0
			17	15
Heated 56°–60° thirty minutes*.	1–20,000 gentian violet, 1		1	1
	No gentian violet, 1		1	1

* Three samples not tested.

with gentian violet lactose broth and negative tests with plain lactose broth; in only one of these was *B. coli* isolated and the presumptive test was negative up to the fourth day. In the other two cases *B. coli* was not isolated from the sample; had gentian violet not been omitted from the lactose agar plate in these two cases *B. coli* might have been isolated as in the other case with its use. In no case did a sample yielding *B. coli* in the presumptive test without gentian violet fail to yield this organism from the presumptive test with gentian violet.

We note that in 5 positive presumptive tests in gentian violet lactose broth from which streaks made on plain lactose agar plates

showed acid colonies, *B. coli* could be isolated but twice. The acid colonies in each of these cases proved to be gram positive cocci. In 3 samples, plates of which were acid after streaking from positive presumptive tests in plain lactose broth, gram positive cocci were responsible in each case for the acid; gas forming aerobes could not be isolated, but from one came a gram negative coliform bacillus giving acid on litmus lactose agar and not liquefying gelatin. This and one other sample of the three yielded *B. coli* from the presumptive test in gentian violet lactose broth plated on plain litmus lactose agar. From 18 samples tested in lactose broth with 1–20,000 gentian violet and streaked out on gentian violet litmus lactose agar *B. coli* was isolated in each case; from 17 samples tested in plain lactose broth and streaked out on gentian violet litmus lactose agar *B. coli* could not be isolated in two cases. In one of them only a large Gram positive bacillus, acidifying the plate and apparently not completely inhibited by the gentian violet at a concentration of 1–100,000, was isolated; it did not produce gas from lactose and the subculture from the first presumptive test into a second showed that we had lost the gas former; *B. coli* was isolated from the presumptive test of this sample in lactose broth with 1–20,000 gentian violet. In the other only streptococci appeared on the plate, which was acidified by them; they too were not inhibited by the weaker concentration of gentian violet in the plate and the gas former was lost when the second presumptive test was made; *B. coli* was isolated from the presumptive test on gentian violet lactose broth in this case also.

The single heated sample showing gas in the presumptive test yielded *B. coli* from the lactose broth with, as well as without, gentian violet. The organism had apparently been able to survive the heating. We cannot of course be certain that sporulating anaerobes were absent from this specimen any more than we can be sure that they were absent from the unheated specimens yielding gas in the presumptive test.

From a comparison of our work on water with that on milk we are inclined to conclude that the so-called "attenuated" *B. coli* is less frequent in milk than in water; a larger proportion

of successful isolations came from the first presumptive test. We are not at all sure what constitutes "attenuated" *B. coli*, whether these are organisms that have temporarily lost their ability to ferment lactose or whether they have lost only their ability to ferment lactose aerobically but not anaerobically, that is to say, under a reduced oxygen pressure. In water work we and others have frequently isolated aerobic non acidifying colonies from litmus lactose agar plates which form gas and acid in the fermentation tube, though usually slowly; this seems to point to the latter condition. In our milk studies no such cases were encountered, due we believe to the fact that colon bacilli recovered from milk on lactose agar are in full possession of their lactolytic power because of the recent activity of that function in the milk itself.

Our main conclusion is that while we are not justified as yet in claiming that the addition of gentian violet to lactose broth will enable us to say in *every* case that *B. coli* is present when gas is formed, yet its use certainly does not interfere with the demonstration of *B. coli*; indeed it distinctly favors such demonstration. Further, the use of 1–100,000 gentian violet in the litmus lactose agar plate does not interfere with the growth or acid producing properties of the organism; rather it eliminates to a large degree the masking of acid produced by *B. coli*, through the inhibition of alkali forming, proteolytic, non-lactolytic, Gram positive sporulating bacilli of the hay bacillus group, not to mention the advantage of preventing spreading growths due to these organisms. Moreover the inhibition of acidifying cocci is of distinct advantage in the search for *B. coli*.

We are frankly surprised not to have demonstrated sporulating anaerobes in milk heated for thirty minutes to 56 to 60°C. It may well be that larger samples, say 10 cc., such as we have used in our water examination would give positive results. Another explanation may be mentioned tentatively, namely, that the presence of lactose in the milk has caused lactolytic sporulating anaerobes to vegetate so that they would be as susceptible to heating as *B. coli*, since various investigators such as Simonds (1915), Hibler (1908), and others, as well as our

own experience have taught that the presence of fermentable carbohydrates inhibits sporulation of anaerobic bacteria. Aside from these forms, however it is to be expected that certain sporulating anaerobes incapable of fermenting lactose would gain access to milk and, theoretically at least, there should be no inhibition of sporulation in their case. This particular phase of the problem will have to be attacked in another way.

REFERENCES

A. P. H. A. 1916 Provisional report to the laboratory section of the American Public Health Association. Amer. J. Pub. Health, **6**, 1315.

Ayers 1917 The significance of colon bacillus in milk. Abstracts of Bact., **1**, 52.

Breed and Brew 1916 Counting bacteria by means of the microscope. N. Y. Agric. Exp. Sta. Tech. Bull. no. 49.

Cal. 1915 State Dairy Bureau. Laws of California relative to production and standards of dairy products.

Hall and Ellefson 1918 The elimination of spurious presumptive tests for B. coli in water by the use of gentian violet. J. Bact., **4**, 329.

Harding 1917 Bacterial count as an index of cleanliness in milk. Abstracts of Bact., **1**, 53.

Hibler 1908 Untersuchungen über die pathogenen Anäeroben. Gustav Fischer in Jena.

Sherman 1916 The advantages of a carbohydrate media in the routine bacterial examination of milk. J. Bact., **1**, 481.

Simonds 1915 Studies in Bacillus welchii. Monograph no. 5, Rockefeller Inst. for Med. Res.

ON THE VALUE OF THE PETROLEUM-ETHER METHOD FOR THE ISOLATION OF B. TYPHOSUS FROM FECES

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The use of petroleum-ether in the isolation of typhoid bacilli from feces was suggested by Bierast (1914). He added a finger's breadth of petroleum to a fecal suspension in broth and shook the mixture at intervals. After allowing this to stand in a cool place for sixteen hours, he plated the sediment or some of the broth from the bottom of the container. He reported that 2 out of 23 specimens were positive only by this method. The work was interrupted because he developed typhoid fever. Jaffe (1915) had favorable results with 24 specimens, as follows:

NUMBER	DIRECT PLATING	AFTER SHAKING, ETC.
5	–	–
5	+	+
0	+	–
4	–	+

He calls attention to the danger of the method and reports a case of typhoid fever due to its use.

Hall (1915) working with the method found that the activity of the different fractions obtained by the distillation of crude petroleum varied inversely with the boiling point. He found that the fraction with a boiling point about 40°C. gave the best results. This fraction would contain a large proportion of pentane (C_5H_{12}) and for convenience we shall refer to it as such.

Hall modified the Bierast procedure as follows. To a moderately heavy suspension of feces in broth is added one-half its

volume of pentane. Close the bottle tightly with a rubber stopper and shake for one-half hour in a shaking machine. The container is then allowed to stand for one and one-half to two hours at room temperature, the bottom fluid or sediment removed with a pipette and plated. His results may be summarized as follows:

SPECIMENS EXAMINED	DIRECT PLATING	PENTANE SHAKING
69	Positive, 13	Positive, 13
	Positive, 1	Negative, 1
	Negative, 7	Positive, 7
	Negative, 48	Negative, 48

Thus, while one specimen positive on direct plating was negative after shaking, 7, or 33 per cent of the total positives, were positive only after shaking.

These results were so favorable that we investigated the method. Unless otherwise stated, we have followed the method of Hall.

Our first attempts were made with stools from typhoid carriers and with greatly diluted broth cultures of recently isolated typhoid strains. The shaking was for one-half hour, and after shaking, the emulsions were allowed to stand for two hours before plating on the Endo medium. The results may be roughly illustrated by the following examples:

MATERIAL	DIRECT PLATING	AFTER SHAKING
Stool suspension	Positive—5 per cent typhoid	1 typhoid colony
Stool suspension	Positive 4,000 total colonies; 75 per cent typhoid	100 total colonies; 90 per cent typhoid
Stool suspension	Positive 5,000 total colonies; 5 per cent typhoid	200 total colonies; 1 per cent typhoid

Broth suspensions of several cultures (pure) yielding by direct plating from 400 to 5000 colonies per plate, after shaking, and then plating an equivalent amount of material, gave no colonies.

While, with the one stool cited above, there was a relative increase in the proportion of typhoid to total flora, it would

obviously be dangerous to expose stools less rich in typhoid to this marked absolute decrease in typhoid colonies. We next tried shortening the time of shaking, and found that in no case did the proportion of typhoid increase on shaking longer than ten minutes, whereas the total number recovered frequently took an alarming drop, and in some cases none was recovered after thirty minutes. It was also determined at this time, that more typhoid bacilli were viable after one than after two hours' standing. The results with this modified technique—that is, time of shaking reduced to ten minutes, and of standing to one hour—may be summarized briefly as follows.

TOTAL EXAMINED	DIRECT PLATING	AFTER SHAKING
12	Positive, 10	Positive, 10
	Positive, 1	Negative, 1
	Negative, 1	Positive, 1

These stools were quite rich in typhoid. Of those in which bacilli were found both before and after shaking, the percentage of typhoid to total flora was increased in three and decreased in seven cases. In 3 of the 7, less than 10 per cent of the number of colonies on the direct plates developed after shaking. In the case of the 3 specimens showing relative increase, 2 of the 3 specimens showed loss of typhoid bacilli, in 1 case to 3 per cent of the original number. The stool which gave positive results only on direct plating had 75 per cent of typhoid (total colonies 900) and yielded 150 non-typhoid colonies after shaking. That in which bacilli were found only after shaking had 6000 colonies on two badly diffused direct plates, which were reduced on shaking to 400, of which 20 were typhoid. In other words, the tendency, even with the reduction of exposure, is to a rapid reduction of the number of typhoid bacilli with which a relative increase compared with the accompanying flora is not necessarily associated.

While these experiments were under way, we conducted a number of tests with pure cultures of recently isolated strains of typhoid, designed to develop a technique more favorable than

that which we were employing. First, we determined that an emulsion of typhoid in broth was not more susceptible to the action of pentane than a similar emulsion in either a normal stool suspension, or in the same suspension sterilized by steaming for one-half hour. Thus:

Summary of reactions of several strains: shaken ten minutes, stood one hour

Direct plating	*After shaking*
In broth 2500 colonies	0
In stool suspension 2500 colonies, 95 per cent typhoid	0
In sterilized stool suspension 2500	0

On attempting to find the approximate percentage of typhoid bacilli which would survive shaking alone, we found wide variations between individual strains. With some, 250 colonies per drop were completely lost on shaking; with others, the numerical reduction varied from 40 to 98 per cent. With this reduction in numbers, there was frequently a concomitant reduction in the size of the colonies developing after shaking. It was thought that the physical effect of shaking might be responsible, but on shaking without pentane (with equivalent saline dilution) practically no reduction occurred, whereas, in this case, 5000 colonies per loop were killed by pentane. It was thought that the bacilli might be contained within the supernatant pentane rather than in the subjacent broth, but culture from the pentane in two cases where the broth yielded from 3 to 25 colonies per drop, showed it to be sterile.

We then investigated more accurately the effect of standing after shaking. The results with several strains were:

DIRECT PLATING	IMMEDIATELY AFTER SHAKING	STOOD 15 MINUTES	STOOD 30 MINUTES	STOOD 45 MINUTES	STOOD 1 HOUR
5,000*	3,000	3,000	2,000	2,000	1,800
3,000	600	160	65	27	25
1,400	400	21	12	12	3

* Strain (in other tests) shows unusual resistance to pentane.

In a final series of stool examinations, small amounts of carrier stool were added to normal stools so as to give mixtures poor in

typhoid. Obviously, it is only with this class of material that the method, if of value, will aid in diagnosis. In order to determine more accurately the initial number of typhoid bacilli, we incorporated the use of the brilliant green medium described by Krumwiede, Pratt and McWilliams (1916), which has proved extremely successful in the isolation of typhoid bacilli from feces. As preliminary tests had shown that in stools as well as in broth cultures, the typhoid bacilli were more or less rapidly lost on standing after the shaking was over, we plated immediately after shaking ten minutes with pentane, and obtained the following results.

TOTAL EXAMINED	DIRECT ENDO	DIRECT GREEN-DYE	ENDO AFTER SHAKING
50	Positive, 21 Negative, 7 Negative, 9 Negative, 13	Positive, 21 Positive, 7 Positive, 9 Negative, 13	Positive, 21 Positive, 7 Negative, 9 Negative, 13
Total positives	21	37	28
Percentage positive..........	42.0 per cent	74.0 per cent	56.0 per cent
Percentage positive compared with total number positive..................	56.7 per cent	100.0 per cent	75.6 per cent

These results indicate that the method with very short exposure has some value as compared with the direct plating on a non-restraining medium (Endo) alone. The general tendency to reduction in the number of typhoid bacilli, not necessarily associated with a markedly greater decrease in the fecal flora, was again observed in this series. On the whole, the data presented indicate that the method is not a reliable one for the relative enrichment of typhoid bacilli in stools. Compared with direct plating on a differential medium (brilliant green agar) the method is evidently inferior.

A report by Schuscha (1916) recently available only in an abstract indicates that his results with the method have not been more satisfactory than with direct plating.

We might add that the method of pentane shaking is unsuited to routine laboratory work. The pentane itself is highly inflam-

mable, and the containers are protected from leaking during agitation in the machine only with difficulty. Two workers abroad have developed typhoid fever while using the method and we feel that there is a large element of danger in its use because of the tendency for the contents of the bottles to blow out when opened, due to the highly volatile pentane.

CONCLUSIONS

The petroleum-ether method for the detection of *B. typhosus* has no advantage over direct plating on a medium which restrains the growth of the associated fecal types. With short periods of exposure, it may be successful where direct plating on Endo alone fails. There is a strong tendency to reduction of the number of *B. typhosus*, not necessarily associated with a greater reduction of the accompanying fecal bacteria. The danger of infection to those using the method, however, is sufficiently serious to warrant its condemnation, especially as it offers no advantage over direct plating on differential restraining media.

REFERENCES

Bierast 1914 Centralbl. f. Bakt. Orig., **74**, 348.
Hall, H. C. 1915 Berl. Klin. Woch., No. 52.
Jaffe 1915 Wien, Kl. Woch. No. 16, 418.
Krumwiede, Pratt and McWilliams 1916 J. Inf. Dis., **18**, 1.
Schuscha 1916 Centralbl. f. Bakt. Orig., **78**, 226. Abstract in Bull. Inst. Pasteur, May 15, 1917.

BACTERIAL NUTRITION: FURTHER STUDIES ON THE UTILIZATION OF PROTEIN AND NON-PROTEIN NITROGEN

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It has been demonstrated by independent investigators (Bainbridge, 1911, and Sperry and Rettger, 1915) that bacteria are unable to bring about the decomposition of native or unchanged proteins by direct action. These proteins can serve as food material for bacteria only through the agency of cleavage-producing substances like acids, alkalies and proteolytic enzymes. Even the most active gelatin-liquefying and putrefactive organisms die from inanition in a medium which contains egg albumin, serum albumin or the vegetable protein, edestin, as the only possible source of nitrogen.

It has been shown further by Rettger, Berman and Sturges (1916) that egg albumin which has been coagulated by heat still retains its resistance to direct bacterial action, and that the proteoses and the higher or more complex polypeptids or peptone fractions of Witte's "peptone" apparently show the same indifference to microbic influence.

The existence of an erepsin-like enzyme in certain bacteria was clearly indicated in the experiments of the writers (1916) which have been conducted on nitrogen utilization in Witte's peptone. The peptolytic enzyme elaborated by the Coli-typhi-paratyphi group and by other gelatin-non-liquefying organisms studied is decidedly weak, and differs further from erepsin of the animal body by its inability to attack casein. At least two weeks were required in the earlier tests to bring about a noticeable reduction in the amount of biuret-giving substances in Witte's

peptone, and even after four weeks of incubation the decrease was small, with one exception.

The work upon which this paper is based was a continuation of the investigations to which reference has been made, and dealt with the following topics:

a. The behavior of bacteria towards coagulated egg albumin.
b. The utilization of different brands of commercial peptone.
c. The utilization of "purified" proteose.
d. The utilization of gelatin and casein.
e. The utilization of the products of Witte's peptone and of casein obtained by partial digestion with trypsin.

THE BEHAVIOR OF BACTERIA TOWARDS COAGULATED EGG ALBUMIN

The results obtained were in strict accord with those of the earlier experiments. A number of additional organisms were employed in the later tests, as for example *Pseudomonas fluorescens*, *Bacillus cloacae* and new strains of *Proteus vulgaris*, *Bacillus subtilis* and *Bacillus coli*.

But one conclusion can be drawn from the results of the combined tests. Coagulated egg albumin, like the unheated and unchanged proteins previously studied, resists the action of even the most active proteolytic bacteria, and can be utilized as food only after at least partial disintegration or cleavage by enzymes or other protein-destroying agents. In all of these experiments the methods employed were the same as those described by Sperry and Rettger (1915).

The individual cell, whether animal, plant or bacterial, requires simple forms of nitrogen, at least until it may produce its own enzymatic substance. The nourishment of bacteria is, as Distaso (1916) has well stated, a passive one, and depends upon substances which can readily pass through the cell walls and be absorbed by the protoplasm. This view is further supported by the experiments which follow.

THE UTILIZATION OF DIFFERENT BRANDS OF COMMERCIAL PEPTONE

Laboratory culture media with few exceptions contain "peptone" as their main nitrogenous ingredient. At the present time various brands of this soluble protein and amino acid complex are available in the open market. The most popular and best known is the Witte product, which until quite recently was used almost exclusively. Chemical analyses indicate that this product has been incorrectly labelled. It has been more than thirty years since Kühne and Chittenden (1884) published their results of the analysis of Witte's peptone. They found the preparation to be rich in albumose. The uniformity of this brand as to composition has made it the universal standard for many years.

At the present time several American brands of peptone are on the market which bid fair to take the place of the Witte product, but none of which have as yet attained the individual prominence of the foreign brand. Chemical tests have shown that the American brands differ to a considerable extent in their composition.

Several methods are available for the study of "peptone" utilization. A brief review will show, however, how far from adequate, with few exceptions, these methods are, and how they may readily lead to wrong conclusions.

Abderhalden, Pincussohn and Walther (1910) employed optical methods in their investigation of the action of certain pathogens on peptone. Since the optical properties of the products of decomposition may have a neutralizing effect, the use of such a test is open to error. The presence of meat extract in a medium may also interfere.

Crabill and Reed (1915) studied the biochemical activities of microörganisms by pouring the test media into Petri dishes and inoculating them with the bacteria under investigation. The digestion and utilization of the medium could thus be determined by direct observation of the plates. Like the auxanographic method of Eijkmann (1901), this is a qualitative procedure and consequently not suitable for the present problem.

The quantitative determination of ammonia production in nutrient broth has been extensively made use of in estimating the comparative ability of various groups of bacteria to attack protein. Among the staunch supporters of this method are Kendall and his co-workers. Caution is necessary in drawing conclusions from the results of this test, for an increase in the ammonia content of a medium does not necessarily indicate that the protein has been attacked. There are other sources of ammonia in nutrient bouillon besides albumoses and peptones, as for example amino acids and extractive substances.

The biuret test was employed in these experiments, since experience in the earlier work had proven its merit. It is of value in demonstrating the disappearance by digestion or otherwise of protein in a fluid medium. Peckham (1897) used this test to show that *B. coli* is unable to utilize completely the protein present in ordinary nutrient media. Vernon (1904) was the first, however, to demonstrate the value of the biuret method as a quantitative measure of peptone digestion, in his study of the action of erepsin upon commercial peptone.

The formol titration method of Sörensen (1908) was employed in conjunction with the biuret test. This method has already been applied to bacteriological problems. The titration figure is a measure of the amount of primary amino acids present in a medium. Rice (1915) claims to have found the Sörensen more satisfactory than the Van Slyke method for the determination of free amino nitrogen.

The biuret test was employed in essentially the same manner as described by Vernon (1904) and as given in a previous paper from this laboratory (1916). The Sörensen method was conducted as follows. Five cubic centimeters of the medium to be tested were mixed with 50 cc. of tap water and neutralized with N/20 NaOH or HCl. Two cubic centimeters of neutral formaldehyde were then added and the solution again neutralized, with phenolphthalein as the indicator.

In the tables the formol titration values are recorded as the number of cubic centimeters of N/20 NaOH required for 100 cc. of the test medium. In the column under 'Reaction' a similar plan has been followed.

The first part of this investigation involved a study of the utilization of Witte's peptone by different types of bacteria. In later experiments other brands of peptone were investigated. The test media were, as a rule, simple nutrient broths such as are used for routine work in the laboratory. These solutions were always transferred to large test tubes in amounts of 10 cc. The water lost by evaporation was accurately replaced after the incubation period, and preliminary to the testing.

Witte's peptone

In tables 1, 2 and 3 are recorded the results of the experiments on the utilization of Witte's peptone. It will be seen that each of the organisms studied is capable of reducing the amount of biuret-giving substances, though for the gelatin-non-liquefying organisms at least a week's incubation is required to show any

TABLE 1

Showing the utilization of Witte's peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	12.0	18.0	1.0
Subtilis group, average 3 members	*3.3*	49.0	0.0
B. prodigiosus	*4.0*	42.0	0.0
P. vulgaris, average 2 strains	*5.0*	52.0	0.0
P. mirabilis	*4.0*	62.0	0.30
Staph. aureus, 3 strains	*2.0*	47.0	0.55
Staph. albus	*4.0*	52.0	0.50
B. cloacae	*4.0*	52.0	0.40
B. typhi	2.0	32.0	0.65
B. paratyphi A	0.0	32.0	0.70
B. coli, average 4 strains	1.0	43.0	0.75
B. diphtheriae, average 2 strains	5.0	37.0	0.60

Medium employed: 1 per cent peptone, 0.25 per cent Liebig's beef extract, and 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

Note: When results with similar organisms do not differ widely they are averaged for the sake of brevity.

The standard biuret figure for the control is given as 1.0 in all of the tables. According to this system a reduction of 40 per cent in the amount of biuret-giving substances is recorded as 0.60, and one of 100 per cent as 0.0.

Reaction of culture fluids is given in the first column. The results are stated in terms of N/20 acid or alkali. The italic figures denote alkaline reaction.

diminution in these substances. Most of the gelatin liquefiers were able to utilize the proteins completely within a period of three weeks. *B. subtilis*, *B. cereus*, *B. prodigiosus* and *Proteus vulgaris* were especially active in this respect. On the other hand, the non-liquefiers exerted but a weak peptolytic action, the average amount of biuret-giving substances acted upon being less than 30 per cent of the total. Among the gelatin

TABLE 2

Weekly tests showing utilization of Witte's peptone

ORGANISMS	BIURET TEST				SÖRENSEN TEST			
	1 week	2 weeks	3 weeks	4 weeks	1 week	2 weeks	3 weeks	4 weeks
(1) Without beef extract								
Control	1.0	1.0	1.0	1.0	16.0	16.0	16.0	16.0
B. subtilis	0.9	0.0	0.0	0.0	62.0	104.0	110.0	90.0
Staph. aureus	1.0	0.9	0.9	0.8	20.0	34.0	40.0	46.0
B. cloacae	1.0	0.8	0.8	0.8	28.0	36.0	44.0	52.0
B. typhi	1.0	1.0	0.9	0.9	14.0	22.0	26.0	32.0
B. coli, average 2 strains	1.0	0.8	0.8	0.8	20.0	31.0	42.0	52.0

Medium: 1 per cent peptone, and 0.5 per cent NaCl.

ORGANISMS	BIURET TEST				SÖRENSEN TEST			
(2) With beef extract								
Control	1.0	1.0	1.0	1.0	24.0	24.0	24.0	24.0
B. subtilis	0.85	0.3	0.0	0.0	84.0	108.0	130.0	112.0
Staph. aureus	0.9	1.0	1.0	0.7	34.0	48.0	54.0	76.0
B. cloacae	0.9	0.7	0.6	0.7	60.0	82.0	92.0	78.0
B. typhi	1.0	1.0	0.9	0.9	26.0	34.0	42.0	48.0
B. coli, average 2 strains	1.0	1.0	0.8	0.75	30.0	44.0	54.0	54.0

Medium: 1 per cent peptone, 0.25 per cent Liebig's beef extract and 0.5 per cent NaCl.

liquefiers *Staphylococcus aureus* and *St. albus* and *B. cloacae* also were comparatively weak.

Table 3 shows that different salts used in medium B do not affect the course of decomposition of the peptone, as compared with the plain nutrient broth.

The small decrease in the biuret-giving substances during the first week of incubation, brought about by the gelatin liquefiers,

as seen in table 2, is of special interest, and would indicate that even strongly proteolytic organisms utilize the simpler nitrogenous substances during their earlier development, thus sparing the more complex substances until the former are exhausted and until abundant enzyme production has taken place.

The formol titration results are of much interest in connection with the other figures. It is quite apparent that an increase in

TABLE 3

Showing utilization of Witte's peptone with and without the addition of certain inorganic salts.

ORGANISMS	MEDIUM A*		MEDIUM B†	
	Biuret test	Sörensen test	Biuret test	Sörensen test
Control	1.0	10.0	1.0	14.0
B. subtilis	0.0	52.0	0.0	48.0
P. vulgaris	0.0	58.0	0.0	46.0
Staph. aureus, average 3 strains	0.65	35.0	0.65	37.0
B. cloacae	0.5	46.0	0.5	50.0
B. typhi	0.75	22.0	0.7	22.0
B. paratyphi A	0.7	22.0	0.7	30.0
B. paratyphi B‡	0.85	20.0	0.9	24.0
B. bronchisepticus	0.85	12.0	0.9	14.0
B. coli, average 4 strains	0.75	22.0	0.75	27.0
B. diphtheriae, average 2 strains	0.7	23.0	0.75	28.0

* Medium A: 0.5 per cent peptone, 0.25 per cent Liebig's beef extract and 0.5 per cent NaCl.

† Medium B: Same as A plus 0.1 per cent K_2HPO_4, 0.02 per cent $MgSO_4$ and 0.01 per cent $CaCl_2$.

Note: Incubation at 30°C. for three weeks.

the titration value does not necessarily run parallel with the results of the biuret tests.

Other brands of commercial peptone

The other peptones tested were all of American manufacture, namely the Digestive Ferments Company, Parke, Davis and Company, Armour, Eimer and Amend, and the Arlington Chemical Company (aminoids) brands. The results of these experiments are given in tables 4, 5, 6, 7, and 8.

A marked difference in the degree of utilization of the protein material in these different products was observed. The Digestive Ferments Company peptone (Difco) showed approximately the same degree of resistance to bacterial attack by the gelatin-non-liquefying bacteria and the intermediate group of organisms (*Staph. aureus* and *Staph. albus* and *B. cloacae*, as the Witte. On the other hand, "aminoids" and the Eimer and Amend product seem to have undergone the greatest amount of degradation of protein or the more complex biuret-giving substances in the process of manufacture, according to the biuret reduction figures. The "aminoids"[1] brand was reduced by *B. coli*, *B. typhi* and

TABLE 4

Showing the utilization of Digestive Ferments Company peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	16.0	32.0	1.0
Subtilis group, 3 strains	7.5	64.0	0.0
B. prodigiosus	8.0	62.0	0.0
P. vulgaris, 3 strains	5.0	50.0	0.2
Staph. aureus and *albus*, 4 strains	1.5	50.0	0.77
B. typhi and *B. paratyphi A* and *B*	2.0	44.0	0.73
B. coli, 4 strains	1.5	44.0	0.7
B. diphtheriae, no. 8	8.0	42.0	0.75

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

B. paratyphi from a biuret value of 1.0 to 0.3 and 0.4, and by *B. diphtheriae* to 0.1 and 0.3, as compared to 0.4 and 0.5 for the Eimer and Amend product. The remaining brands occupy an intermediate position between these two extremes.

The experimental evidence at hand indicates that most of the nitrogen present in commercial peptones is in a form too complex to be utilized directly. *B. subtilis*, *B. prodigiosus*, *P. vulgaris* and the closely related forms are able to digest the different peptones completely, whereas those organisms which are not

[1] Two brands of "aminoids" have been used in this laboratory. The one contains an appreciable amount of biuret-giving substances, while the other is biuret-free. The former was employed in these experiments.

known to elaborate proteolytic or gelatin-attacking enzymes, as for example *B. coli* and *B. typhi*, exert but little influence on the biuret-giving substances of some of the peptones, particularly Witte's.

Commercial peptones are now regarded as being a mixture of polypeptids of all degrees of complexity. It is probable that the peptone fraction of these products contains polypeptids varying in composition from the simplest dipeptids to those which are relatively very complex. The former are more easily utilized as food by the bacteria than the higher or more permanent forms,

TABLE 5

Showing the utilization of Parke, Davis and Company peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	28.0	1.0
Subtilis group, 3 members	*3.5*	62.0	0.0
B. prodigiosus	*6.0*	48.0	0.0
P. vulgaris, 2 strains	*3.0*	56.0	0.0
P. mirabilis	10.0	48.0	0.7
Staph. aureus and *albus*, 4 strains	7.5	53.5	0.6
B. cloacae	*2.0*	54.0	0.4
B. typhi and *B. paratyphi A*	*4.0*	54.0	0.6
B. coli, 4 strains	*2.0*	52.0	0.6
B. diphtheriae, 2 strains	6.0	54.0	0.5

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

and hence disappear from solutions first.[2] As even some of the very simple polypeptids are known to give the biuret color reaction, we may find here a possible explanation of the slow, gradual but limited reduction in the biuret figures which the non-proteolytic organisms studied brought about. It is the amino acids and other simple nitrogenous substances, however, to which commercial peptone chiefly owes its value as food for bacteria.

It has been shown repeatedly that meat extract in nutrient broth, etc., contains valuable food substances, and that it is for

[2] Sasaki (1912) has shown that simple polypeptids are attacked by bacteria.

this reason a very important ingredient of the ordinary laboratory media. The older belief in a mere "stimulating effect" of meat extract on bacteria is unscientific and untenable. The real food value lies in the amino acids (primary and secondary) and in the so-called "extractives, kreatin, kreatinin, hypoxanthin, etc."

The behavior of *St. aureus*, *St. albus* and *B. cloacae* in the peptone was surprising. These gelatin-liquefying organisms were able to attack the biuret-positive substances to only a

TABLE 6

Showing utilization of Armour's peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	30.0	1.0
Subtilis groups, 3 members	5.0	60.0	0.0
B. prodigiosus	4.0	54.0	0.0
P. vulgaris, 2 strains	1.0	56.0	0.0
P. mirabilis	8.0	66.0	0.4
Staph. aureus and *albus*, 4 strains	7.0	60.0	0.5
B. cloacae	2.0	56.0	0.5
B. typhi, and *B. paratyphi A* and *B*	5.5	50.0	0.65
B. coli, 4 strains	3.5	52.0	0.57
B. diphtheriae, 2 strains	7.0	54.0	0.5

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

slightly greater extent than the non-liquefiers. This observation is in accord, however, with those of Malfitano (1903) who pointed out a distinct difference between the albumolytic and the gelatinolytic properties of an organism. In his work with *B. anthracis* he showed that the addition of chloroform influenced the albumin-digesting, but not the gelatin liquefying property. A further review of the literature (Mavrojannis, 1903, and Jordan, 1906) justifies the conclusion that the ability of an organism to liquefy gelatin is no indication of its proteolytic power.

TABLE 7

Showing the utilization of Eimer and Amend peptone

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	24.0	1.0
Subtilis group, 3 members	6.0	49.0	0.0
B. prodigiosus	*2.0*	48.0	0.0
P. vulgaris, 2 strains	7.0	42.0	0.0
P. mirabilis	14.0	42.0	0.3
Staph. aureus and *albus*, 4 strains	12.0	38.0	0.48
B. cloacae	8.0	38.0	0.4
B. typhi, and *B. paratyphi A* and *B*	7.0	35.0	0.45
B. coli, 4 strains	8.0	37.5	0.43
B. diphtheriae, 2 strains	10.0	39.0	0.43

Medium: 0.5 per cent peptone, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

TABLE 8

Showing the utilization of "aminoids" of Arlington Chemical Company

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	20.0	42.0	1.0
Subtilis group, average 3 members	*4.0*	57.0	0.0
B. prodigiosus	*4.0*	54.0	0.0
P. vulgaris, 2 strains	*3.0*	46.0	0.0
P. mirabilis	12.0	64.0	0.0
Staph. aureus, 3 strains	9.0	59.0	0.3
Staph. albus	10.0	64.0	0.3
B. cloacae	4.0	46.0	0.25
B. typhi, and *B. paratyphi A* and *B*	*2.5*	56.0	0.3
B. coli, 4 strains	*1.0*	51.0	0.35
B. diphtheriae	9.0	52.0	0.2

Medium: 0.5 per cent aminoids, 0.25 per cent beef extract, 0.5 per cent NaCl Incubation at 30°C. for three weeks.

THE UTILIZATION OF PURIFIED PROTEOSE

Proteose was obtained from commercial "peptone" by salting out with ammonium sulphate. The first precipitate was dissolved in water and the proteose reprecipitated with the sulphate. After dialysis for several days the proteose solution was converted into a test medium by the addition of Liebig's

extract and sodium chloride. The amounts of the different substances in solution are stated in the following tables.

Another method of at least partial purification of proteose consisted in dialyzing a concentrated solution of "peptone." It was to be expected that the simpler biuret-giving substances which in their aggregate constitute what may be called the peptone fraction of the commercial "peptone" would pass through the parchment membrane, leaving the more complex ingredients, the proteose portion in particular, behind. The process of dialysis was allowed to continue for from six to eight days. In order to prevent bacterial decomposition of the dialysate it

TABLE 9

Showing the utilization of "purified" proteose

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
Control	6.0	10.0	1.0
B. subtilis and *B. ramosus*	5.0	78.0	0.0
B. prodigiosus	10.0	68.0	0.0
P. vulgaris, 2 strains	14.0	10.0	0.9
P. mirabilis	16.0	12.0	1.0
Staph. aureus and *albus*, 4 strains	11.0	11.0	0.95
B. cloacae	2.0	22.0	0.95
B. typhi and *B. paratyphi A*	19.0	11.0	1.0
B. coli, 2 strains	12.0	12.0	1.0
B. coli, 1 strain	20.0	10.0	0.95

Medium: 0.8 per cent proteose, 0.25 per cent beef extract, 0.5 per cent NaCl. Incubation at 30°C. for three weeks.

was boiled for a short period each day, and the water in the jar was frequently changed.

The experiments with the proteose media were conducted in the same manner as those in which the commercial peptones were employed. The results of the first series of tests are recorded in table 9. The beef extract was added to encourage bacterial development. Only those culture flasks which gave positive evidence of growth were used.

B. subtilis, B. ramosus and *B. prodigiosus* completely destroyed the proteose, as shown by the biuret test. *Proteus vulgaris* and *P. mirabilis* produced only a slight change, owing to their slow

development. The beef extract was not sufficient to supply the initial needs of these organisms for enzyme production. The proteus group is known to find poor growth conditions in synthetic media, particularly the Uschinsky medium. The staphylococcus forms affected the biuret-giving substances but very little, if at all. The members of the Coli-typhi-paratyphi group, furthermore, were unable to attack the purified proteose, during the entire incubation period of three weeks. The Sörensen values strongly support those of the biuret test.

An experiment was conducted to determine whether proteose is attacked in a medium in which it furnishes the sole source of nitrogen. The test solution consisted of approximately 0.25

TABLE 10

Showing the behavior of bacteria in dialyzed Witte's peptone

ORGANISMS	IMMEDIATE	AFTER INOCULATION				REACTION	SÖRENSEN TEST	BIURET TEST
		24 hours	48 hours	6 days	2 weeks			
B. subtilis	11	6	2	2	0	2.0	2.0	1.0
B. prodigiosus	10	x*	x	x	x	2.0	8.0	0.0
B. typhi	16	1	1	2	1	2.0	2.0	1.0
B. coli	16	1	1	1	1	2.0	2.0	1.0
Control						2.0	2.0	1.0

Medium: 0.25 per cent "proteose," 0.5 per cent NaCl. Chemical tests made after incubation of three weeks.

* x indicates too many colonies to count.

per cent proteose and 0.5 per cent NaCl. The medium was employed in small Erlenmeyer flasks in 50 cc. quantities. The technique was essentially the same as in the experiments with native proteins and with coagulated egg albumin. Bacterial counts were made by the plate method, immediately after inoculation and after definite intervals. Biuret and Sörensen determinations were also made. The results are given in table 10. They indicate that proteose resists direct bacterial attack, and thus falls in the same group with the native proteins and coagulated egg albumin.

B. prodigiosus, as in the experiments on native proteins, was able to develop in the test medium. This may be explained by

the ease with which the organism produces its proteolytic enzyme and by the unusual activity of this enzyme when present in very small amount. None of the other bacteria employed was able to increase in numbers in the proteose medium, all of the flasks remaining as clear as the control.

A series of tests was carried out also with the diphtheria bacillus, and the results recorded in table 11. Both strains of the organism used were unable to produce any change in the proteose content, as is shown clearly by the biuret tests, and corroborated by the Sörensen figures. Multiplication of the organisms was entirely at the expense of the meat extract. Whether any of the albumoses are affected by *B. diphtheriae* without losing their biuret-giving property remains undetermined. This

TABLE 11

Showing behavior of B. diphtheriae toward "purified" proteose

ORGANISMS	REACTION	SÖRENSEN TEST	BIURET TEST
B. diphtheriae no. 8, average 8 tests	14.0	28.0	1.0
B. diphtheriae (Y. M. S.), 9 tests	52.0	28.0	1.0
Control, uninoculated medium	32.0	28.0	1.0

Medium: 0.5 per cent proteose, 0.5 per cent beef extract, 0.5 per cent NaCl Incubation at 30°C. for three weeks.

question is of particular interest in view of Hida's (1908) claim that deutero-albumose is essential in diphtheria toxin production.

THE UTILIZATION OF GELATIN AND CASEIN

Although a large number of organisms are known to liquefy gelatin, little has been done to determine whether the gelatin is actually utilized by the bacteria. Experimental evidence was sought in this work as to whether the power to liquefy gelatin carries with it ability to reduce it to its simple components. It has already been pointed out that the gelatinolytic and albumolytic properties of an organism may be distinct. The test medium employed in this investigation contained gelatin as the only known source of organic nitrogen.

The medium used in the first experiment contained 0.25 per cent commercial gelatin and 0.5 per cent NaCl. Liebig's meat extract (0.25 per cent) was added to half of the solution, and the two portions tubed separately in 10 cc. quantities. The tubes were inoculated with various organisms, and after an incubation period of three weeks the reaction and the Sörensen and biuret figures were determined. The results are not given in tabulated form, but may be found as such in the original thesis[3] at the Yale University Library. The reader is also referred to this thesis for other detailed statements and results which have not been incorporated in this paper.

B. subtilis, *B. ramosus* and *B. prodigiosus* were the only organisms studied which were able to completely utilize the gelatin, or to transform it into a-biuretic products. The proteus strains affected it only slightly, while *Staphylococcus aureus* and *B. cloacae* (liquefiers) made no impression whatever on the gelatin, thus showing the same behavior as *B. typhi*, *B. paratyphi*, *B. coli* and *B. Zenkeri*.

The apparent inability of *Proteus vulgaris* and *P. mirabilis* to decompose the gelatin was due to the limitations of the test fluid. Another test medium was prepared which contained opsine, a protein-free commercial product containing a large amount of readily available organic nitrogen for bacterial growth, besides the substances already cited. In this medium *Proteus vulgaris* and *P. mirabilis* were able to utilize from 50 to 70 per cent of the gelatin, as indicated by the biuret tests. The results with the other organisms were the same as in the gelatin solution which contained no opsine. The beef extract aided the metabolism of the proteus forms (not Zenkeri) to a small extent only.

It is difficult to offer a satisfactory explanation of the inability of *Staphylococcus aureus* and *Bacillus cloacae* to affect the decomposition of the gelatin beyond the gelatose stage. The evidence derived from both the Sörensen and biuret figures is sufficiently conclusive to show that the power of an organism to liquefy

[3] Doctorate thesis, Nathan Berman, May, 1917.

gelatin is not necessarily accompanied by the ability to decompose the gelatin and to seize upon it as food.

The digestion of casein has been a subject of study for many years. The observations reported by various investigators are to a large extent contradictory. This may be accounted for in part by a lack of uniformity in the methods. Blumenthal (1896) reported that *B. coli* was able to attack casein, and stated that proteolysis in milk is possible provided the acid formed from the lactose is neutralized as rapidly as it is formed. Taylor (1902) also claimed that *B. coli* could digest casein. He prepared a culture fluid containing purified casein. The inoculated medium was examined after a definite interval for amino acids. Being unable to detect any amino acids, he assumed that the casein was decomposed into proteoses and peptones. From cultures of *P. vulgaris* he was successful in isolating several amino acids, however.

Barthel (1915) studied the digestion of casein by milk-souring bacteria. Rosenow (1913) reported that he was able to obtain an enzyme from a pneumococcus broth culture which could decompose blood serum, but not casein.

In the present investigation the behavior of certan organisms was studied in a medium containing purified casein. The medium contained the following ingredients: 0.125 per cent casein, 0.5 per cent Liebig's meat extract and 0.5 per cent NaCl. The solution was tubed in 10 cc. quantities. A test for unchanged casein was always made with dilute acid. Chief reliance was placed on the biuret and the Sörensen tests.

The behavior of the organisms was the same in this medium as in those containing purified proteose and gelatin. The subtilis members, *B. prodigiosus* and the proteus forms (vulgaris and mirabilis) were able to break down the casein. None of the other organisms studied (*Staphylococcus aureus* and *albus*, *B. typhi A* and *B. B. coli* and *B. diphtheriae*) possessed the ability to affect the casein in the slightest degree, even during three weeks of incubation at 30°C. That *B. coli* and its close allies cannot decompose casein should not be surprising, though the facts as established here are contrary to the observations

of Taylor (1902). It is not at all improbable that what Blumenthal reported as digestion was in reality only a solution of the casein in an alkaline medium.

THE UTILIZATION OF THE PRODUCTS OF WITTE'S PEPTONE AND OF CASEIN OBTAINED BY PARTIAL DIGESTION WITH TRYPSIN

The inability of many organisms to develop in the common laboratory media is due to the absence in these media of sufficient food material which is immediately available for cell nutrition. That a large part of Witte's peptone, as well as gelatin, casein, unchanged native proteins and coagulated egg albumin, is valueless as a source of organic nitrogen, in so far as the large group of gelatin-non-liquefying organisms at least is concerned, has been demonstrated in this investigation. Smith (1897) pronounced plain peptone solution to be a poor culture fluid. He stated that some organisms failed to grow in Witte's peptone. Rivas (1912) found that short tryptic digestion of peptone enhanced its value as a culture medium. Hottinger (1913) who denounced Witte's peptone as unsatisfactory for culture purposes suggested a pancreatic digestion product of meat as a substitute.

Dalimier and Lancereaux (1913) found the protein-free product, "opsine," which is rich in amino acids and other simple nitrogenous substances, to serve as a good culture medium for all of the many organisms studied, including some very fastidious pathogens. These results, with a single exception, were recently corroborated by Robinson and Rettger. Cole and Onslow (1916) suggested a pancreatic digestion product of casein as a good medium, and Distaso (1916) succeeded in obtaining good growths of pathogenic organisms in a solution which contained as its only source of nitrogen serum which had been digested with trypsin.

In the present work solutions of Witte's peptone and of casein were digested with commercial trypsin. The digestion was allowed to continue for five hours at 45°C., after which the liquid was immediately sterilized, and 0.25 per cent beef extract

and 0.5 per cent NaCl added. The media were tubed in 10 cc. quantities, and inoculated with the same organisms used in the preceding tests. The methods of determining the utilization of biuret-giving substances were also the same as those employed throughout the earlier work.

The increased utilization of the predigested peptone was plainly evident. The action of the trypsin almost doubled the amount of nitrogen available for the non-proteolytic organisms. In several instances 65 per cent of the biuret-giving substances of the peptone digestion product was utilized by this group of bacteria, and 35 per cent of the biuret-positive casein digestion product. Aside from the great reduction in the biuret figures, and the high Sörensen titration, the nutrient property of the medium was evidenced by the luxuriance of bacterial growths. It is to be assumed that the large reduction by the gelatin-non-liquefiers in the biuret figure was due to the preponderance of polypeptids of comparatively simple constitution.

GENERAL DISCUSSION AND CONCLUSIONS

The results obtained in the present investigation confirm those of previous experiments, and further justify the assumption that bacteria require food substances which are of simple constitution and whose properties are such that they readily admit of absorption by the bacterial cell. This view is in harmony with that of Loewi (1902), Abderhalden, etc. as applied to animal nutrition. It furnishes an explanation of the inefficiency of the ordinary laboratory culture media, and particularly solutions of Witte's peptone, for rapid and abundant production of bacterial growth.

Commercial peptones are highly complex products, containing, among other things, different albumoses and so-called peptones, amino acids, particularly the primary, and other simple nitrogen-containing substances. No two brands of commercial peptone are of the same composition, nor is it likely that any one product is uniform. Numerous tests have shown that the amino acid content of some products is decidedly

greater than that of others, while the proportion of the albumose and complex polypeptid fraction is correspondingly less.

The presence of a peptolytic enzyme in cultures of coli, typhi and other gelatin-non-liquefying bacteria has apparently been demonstrated. This erepsin-like enzyme is, however, decidedly weak, and further differs from erepsin of the animal body in that it does not attack casein. The decomposition of biuret-giving material in commercial peptone is, without doubt, limited to the substances of relatively simple constitution, or the lower polypeptids. This has been demonstrated by the inability of the non-proteolytic organisms in question to attack "purified" proteose, and in a measure by the peculiar shading off of the biuret color, during prolonged periods of incubation, from the characteristic light purple color obtained with a solution of peptone to the deep purple or violet which is given by proteose and albumin solutions with the alkaline copper sulphate.

It may of course be questioned whether the decomposition of the simple polypeptids of the "peptone fraction" requires any enzyme action, and whether these substances are not sufficiently simple and soluble to be absorbed and utilized directly by the bacterial cell. Such an explanation of the availability of the simple amino acids for bacterial nutrition is quite plausible, since they are very soluble and dialyzable; and further, it would appear improbable that specific enzymes for the many different amino acids are elaborated.

Even the gelatin-liquefying and proteolytic bacteria are slow to make use of the proteoses and peptone in commercial "peptone." Their initial development is at the expense of the simpler nitrogenous compounds, and a reduction in the biuret-giving substances occurs only after a period of preliminary cultural development, this period often extending over at least a day or two. In this preparatory interval the proteolytic enzyme is formed which is necessary in the utilization of the soluble protein material. That the production of the enzyme which attacks gelatin is as a rule a slow process is well shown in the ordinary gelatin liquefaction tests.

The ability to liquefy gelatin is not in itself a proteolytic or even a gelatinolytic function, for some organisms which can liquefy gelatin are unable to carry the change beyond the gelatose stage, and fail to decompose "purified" proteose and casein.

Members of the coli-typhi-paratyphi group of organisms studied are without ability to decompose gelatin and casein. They are unable also to affect the "proteoses" in commercial peptone, and in their reduction of the biuret-giving substances attack and decompose only the simpler of these components, presumably the lower polypeptids; the action on even these relatively simple substances is slow and delayed. *Bacillus cloacae* is not here included in the above group of organisms, though its action in the media containing the above substances is almost, if not entirely, the same as that of *B. coli*, etc.

The value of a cultural medium for bacteria depends on the immediately available food substances which it contains. For this reason it appears quite reasonable that those commercial peptones which contain the largest amount of amino acids and other nitrogenous products of simple composition are most conducive to active and abundant bacterial development, every thing else being equal. This supposition is fully borne out by experiment. The question as to whether such peptones are the most satisfactory for toxin production in a medium requires further and extensive investigation.

The conclusions may be summarized briefly as follows:

1. Bacteria are unable to decompose coagulated native protein when there is no other source of available nitrogen in the test medium.

2. There is reason to believe that purified proteose also is resistant to direct attack by bacteria.

3. Gelatin-non-liquefying bacteria and some of the liquefiers are feeble in their action on Witte's peptone. A much better utilization of the biuret-giving substances occurs in media containing commercial peptone which has been subjected to more extensive digestion in the process of preparation, as for example certain of the American brands.

4. The ability of an organism to liquefy gelatin is no sure indication of its proteolytic properties.

5. Gelatin and casein resemble proteose in their resistance to bacterial attack.

REFERENCES

ABDERHALDEN ET AL. Z. physiol. Chem., vols. 42, 44, 47, 52, 57, 59, 77 and 78.

ABDERHALDEN, E., PINCUSSOHN, L., AND WALTHER, A. R. 1910 Untersuchungen über die Fermente verschiedener Bakterienarten. Z. physiol. Chem., **68**, 471–6.

BAINBRIDGE, F. A. 1911 The action of certain bacteria on proteins. J. Hyg., **11**, 341–55.

BARTHEL, C. 1915 Das kaseinspaltende Vermögen von zur Gruppe Streptococcus lactis gehörenden Milchsäurebakterien. Centralbl. Bakt., Abt. 2, **44**, 76–89.

BERMAN, N., AND RETTGER, L. F. 1916 Bacterial nutrition; A brief note on the production of erepsin by bacteria. J. Bact., **1**, 537–9.

BLUMENTHAL, F. 1896 Ueber die Produkte der bakterischen Zersetzung der Milch. Virchow's Arch., **146**, 65–85.

COLE, S. AND ONSLOW, R. 1916 On a substitute for peptone and a standard nutrient medium for bacteriological purposes. Lancet, **191**, no. 4844, 9–11.

CRABILL, C. H. AND REED, H. S. 1915 Convenient methods for demonstrating the biochemical activity of microörganisms with special reference to the production and activity of enzymes. Biochem. Bull., **4**, 30–44.

DISTASO, A. 1913 Sur la production de l'indol par le B. coli en milieux au tryptophane et sucres. Compt. rend. Soc. Biol., **75**, 200–1.

EIJKMANN, C. 1901 Ueber Enzyme bei Bakterien und Schimmelpilze. Centralbl. Bakt., **29**, 841–8.

HIDA, O. 1908 Ueber die Bedeutung der Peptone für die Bildung des Diphtherietoxins. Z. Hyg. **61**, 273–81.

HOTTINGER, R. 1913 Nachprüfung und Kritik der üblichen Bouillonbereitung. Centralbl. Bakt., Abt. 1, Orig., **67**, 178–206.

JORDAN, E. O. 1906 Experiments with bacterial enzymes. Biological Studies by the Pupils of W. T. Sedgwick, 124–45.

KÜHNE, W., AND CHITTENDEN, R. H. 1884 Ueber Albumosen. Z. Biol., **20**, 11–51.

LOEWI, O. 1902 Ueber Eiweissynthese im Thierkorper. Arch. exp. Path. u. Pharm., **48**, 303–30.

MALFITANO, M. G. 1903 Sur la dissociation pouvoir albuminolytique et du pouvoir gelatinolytique de la protéase charbonneuse. Compt. rend. Soc. Biol., **55**, 843–45.

MAVROJANNIS, A. 1903 Das Formol als Mittel zur Erforschung der Gelatineverflüssigung durch die Mikroben. Z. Hyg., **45**, 108–14.

PECKHAM, A. W. 1897 The influence of environment upon the biological processes of the various members of the Colon group of bacilli. J. Exp. Med., **2**, 549–91.

Rettger, L. F., Berman, N., and Sturges, W. S. 1916 Further studies on bacterial nutrition. The utilization of proteid and non-proteid nitrogen. J. Bact., **1**, 15–33.

Rice, F. E. 1915 Studies on the action of erepsin. J. Am. Chem. Soc., **37**, 1319–33.

Rivas, D. 1912 Studies on indol. Centralbl. Bakt., Abt. 1, Orig., **63**, 547–50.

Rosenow, E. C. 1912 On the toxicity of broth, of pneumococcus broth culture filtrates, and on the nature of the proteolytic enzyme obtainable from pneumococci. J. Inf. Dis., **11**, 286–93.

Sears, H. J. 1916 Studies in the nitrogen metabolism of bacteria. J. Inf. Dis., **19**, 105–37.

Smith, Th. 1897 A modification of the method for determining the production of indol by bacteria. J. Exp. Med., **2**, 543–7.

Sörensen, S. P. L. 1908 Enzymstudien. Biochem. Z., **7**, 45–101.

Sperry, J. A. and Rettger, L. F. 1915 The behavior of bacteria towards purified animal and vegetable proteins. J. Biol. Chem., **20**, 445–59.

Taylor, A. E. 1902 Ueber Eiweisspaltung durch Bakterien. Z. physiol. Chem., **36**, 487–92.

Vernon, H. M. 1904 The peptone-splitting ferments of the pancreas and intestine. J. Physiol., **30**, 330–69.

THE INFLUENCE OF CARBOHYDRATE ON THE NITROGEN METABOLISM OF BACTERIA

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The use of fermentable sugars, alcohols and glucosides in bacteriological culture media has marked a distinct advance in the present methods of studying and classifying bacteria. Chief emphasis has been placed, however, on the question as to whether certain given organisms attack these substances with resultant acid and gas production. Among those who have given their attention to the utilization of carbohydrates and their influence on nitrogen metabolism considerable difference of opinion exists; and a review of the literature shows that further and more definite information on this subject is indeed desirable.

Liborius (1886) observed that the presence of sugar did not prevent liquefaction of gelatin by *Bacillus subtilis*, *Pseudomonas fluorescens* and *Bacillus pyocyaneus*. Winternitz (1892), as the result of animal feeding experiments, came to the conclusion that glucose and lactose inhibit the bacterial decomposition of protein. Glenn (1911) claimed that in a medium containing sugar and protein material the carbohydrate is first attacked, and that the protein is subsequently decomposed, providing the acidity produced in the complete fermentation of the sugar is not too great. According to Glenn, therefore, no protein utilization takes place in the presence of fermentable carbohydrate.

Kendall, Day and Walker (1913) emphasized the preference of bacteria for sugars. According to them nitrogen metabolism in carbohydrate media is at its minimum. Aubel and Colin (1913) observed that fermentable sugars inhibit color production of bacteria. A year later these same authors found that the addition of glucose to a medium lessens or inhibits the pro-

duction of ammonia. Kendall, Day and Walker (1915) failed to demonstrate the formation of an active proteolytic enzyme by *Proteus vulgaris* in either glucose broth or glucose gelatin. The enzyme produced in carbohydrate-free medium, and freed from the bacteria by filtration, liquefied glucose gelatin as readily as plain gelatin, however. According to these workers, the presence of sugar delays the formation of the proteolytic enzyme until the carbohydrate has been used up. Jones (1916) also concluded that the failure of *P. vulgaris* to decompose the protein in a glucose gelatin medium is due to the absence of enzyme formation. He states that acidity plays no part in influencing the behavior of the organism. Sears (1916) concluded that glucose had a sparing action on nitrogen metabolism. Kligler (1916) offers a similar explanation, and states that the proteolytic enzyme of *Proteus vulgaris* is not formed in the early stages of carbohydrate metabolism.

Other investigators observed the influence of sugar on indol production. Baginski (1889) reported that he was unable to obtain an indol test in the presence of lactose. Péré (1892) concluded that a positive test for indol indicated a complete disappearance of sugar. He affirms that carbohydrates are more available than protein, and are utilized first. Distaso (1913) showed that indol formation may occur in a carbohydrate medium containing free tryptophan. *B. coli* was employed as the test organism. According to this writer the more available substances in a medium are used first, and as a consequence indol is not formed in ordinary sugar media. De Graaff (1909) had previously observed the inhibiting effect of glucose on indol production.

Rougentzoff (1913) claimed that the carbohydrates, glucose, fructose and lactose, and the alcohol mannite, which are fermented rapidly by *Bacillus coli*, inhibit indol production. Indol was formed, however, in media containing maltose, sucrose and dulcite. The titratable acidity was taken as an index of the rapidity with which different saccharides were decomposed. Fischer (1915) reported somewhat different results. He observed that glucose alone inhibited indol formation, while lactose, maltose,

galactose and fructose were without effect. He believed that the acid formed played no rôle, since the addition of calcium carbonate to the medium did not influence the results. Fischer concluded that the inhibitive action of the glucose is due to an inactivation of the proteolytic enzyme of the organism. This view is partly in harmony with the theory recently advanced by Homer (1916), according to which a chemical union is formed between the aldehyde group of the glucose and a portion of the tryptophan molecule. The combination is more resistant to attack than free tryptophan.

EXPERIMENTAL

A study of the behavior of certain organisms in glucose peptone broth was first undertaken. The test medium contained peptone (Witte) 0.25 per cent, sodium chloride 0.5 per cent, and glucose 1.0 per cent. The same medium minus the sugar was used for control purposes. The test and control solutions were transferred in 10 cc. quantities to test tubes, and inoculated with the test organisms. At the end of four weeks of incubation at 37°C. the water of evaporation was replaced and biuret tests made according to the method of Vernon (1904). The Sörensen (1908) method for the determination of amino acids was also carried out, as well as the Benedict test for the presence of unchanged sugar. The results are presented in table 1.

Barring one exception, no protein decomposition occurred in the presence of glucose. *Bacillus subtilis* alone was able to digest the peptone in the glucose-containing medium. In every other instance the biuret and Sörensen tests gave the same figures as in the uninoculated controls. The acidity in the glucose tubes was increased, with the exception of the case of *Bacillus ramosus*. Owing to the unfavorable temperature for this organism, *Bacillus ramosus* grew but sparingly. The digestion of the peptone by *Bacillus subtilis*, even though the glucose was not completely fermented, is an indication that a glucose-utilizing organism may secrete a proteolytic enzyme in the presence of a fermentable sugar.

TABLE 1

Showing the effect of glucose on nitrogen metabolism

ORGANISM	REACTION		SÖRENSEN		BIURET		SUGAR	
	C	D	C	D	C	D	C	D
Control uninoculated	4.0	4.0	4.0	4.0	1.0	1 0	−	+
B. subtilis	2.0	6.0	22.0	28.0	0.0	0.0	−	+
B. ramosus	6.0	4.0	14.0	8.0	0.75	1.0	−	+
P. vulgaris	4.0	12.0	8.0	6.0	0.0	1.0	−	+
P. mirabilis	2.0	14.0	8.0	6.0	0.0	1.0	−	+
Staph. aureus	2 0	10.0	4.0	6.0	0.7	1.0	−	+
Staph. aureus, II	2.0	10.0	8.0	6.0	0.7	1.0	−	+
Staph. albus	4.0	10.0	16.0	4.0	0.7	1.0	−	+
B. typhi	0.0	14.0	6.0	4.0	0.8	1.0	−	+
B. paratyphi A	2 0	14.0	6.0	6.0	0.8	1.0	−	+
B. coli	2.0	20.0	4.0	4.0	0.85	1.0	−	+
B. diphtheriae	4.0	6.0	6.0	6.0	0.85	1.0	−	+

The composition of Media C and D is as follows:

Medium C

	per cent
Peptone	0.5
NaCl	0.25

Medium D

	per cent
Peptone	0.5
NaCl	0.25
Glucose	1.0

A more extensive study of the influence of carbohydrates on the protein metabolism of *Bacillus subtilis* was then conducted. Five different media were prepared as follows:

MEDIUM 1	MEDIUM 2	MEDIUM 3	MEDIUM 4	MEDIUM 5
Peptone, 0.5 per cent Beef extract, 0.25 per cent NaCl, 0.5 per cent	1 plus 0.2 per cent glucose	1 plus 0.5 per cent glucose	1 plus 0.2 per cent lactose	1 plus 0.5 per cent lactose

Medium 1 was used as control. The five solutions were transferred to test tubes (10 cc.), each tube but the control being inoculated with a pure strain of *Bacillus subtilis*. A series of fifteen tubes was used for each medium. Tubes from each series were taken from the incubator on alternate days, and tested

as in the preceding experiment. The incubation periods varied from one to twenty-seven days, a constant temperature of 30°C. being employed. The results are not given here in tabulated form,[1] but may be summed up briefly as follows.

The titratable acidity was at no time high. Towards the end of the experiment the reaction in the glucose and lactose tubes was practically the same as that of the uninoculated controls, 10, as compared with 28 and 30, the highest points of acidity. The Sörensen titration increased simultaneously in all of the inoculated media, and in practically the same degree. The biuret tests indicate the same metabolic changes as the Sörensen titration. The peptone was completely utilized in each of the five media before three weeks of incubation had elapsed. The slow rate at which the sugars were fermented is noteworthy. The first evidence of disappearance of the sugars, even in the tubes originally containing only 0.2 per cent of the carbohydrates, occurred after two weeks, while twenty-five days were required for their complete disappearance from the tubes originally containing 0.5 per cent.

A similar study was made with *Bacillus coli* and *Proteus vulgaris*. The details of the experiment were the same as in the previous experiment, except that with these two organisms two additional tests were conducted, namely the indol and methyl red tests. The Salkowski method of determining the presence of indol was employed. The test was made by adding 0.25 cc. of concentrated sulphuric acid to a few cubic centimeters of the fluid under observation, immediately cooling the mixture in cold water, and slowly adding a 0.5 per cent solution of sodium nitrite while the tube was held in an almost horizontal position. This method permits of a minimum amount of diffusion, so that the color is made all the more apparent in the case of a positive test. Difficulty in procuring the Ehrlich aldehyde prevented the use of this agent until near the close of the investigation. A comparative study of the two methods has shown them to be equally reliable in our own hands.

[1] The original tables may be consulted in the doctorate thesis (Berman), a copy of which is in the possession of each of the writers and of the Yale University Graduate School.

The methyl red test of Clark (1915) was employed because it gives an index of the 'true reaction' of the test media. This test has proven itself to be of much value.

The behavior of *Bacillus coli* in the media containing the glucose and lactose is of particular interest. The biuret and Sörensen tests gave essentially the same figures for the control medium (plain peptone broth) as in previous experiments. The indol appeared on the third day, and remained throughout the course of the experiment. The final reaction was practically neutral to phenolphthalein. In the media containing glucose the results were quite different. The hydrogen ion concentration was markedly increased, both glucose media (0.2 per cent and 0.4 per cent) becoming methyl red positive. In the tubes containing 0.2 per cent of glucose the sugar was completely fermented by the third day. Little metabolism seemed to take place after this. The titratable acidity remained high. The Sörensen values were almost the same as for the controls. No indol tests were obtained in the glucose tubes even after four weeks. Although 0.2 per cent glucose was used up in three days, 0.5 per cent was not completely fermented in a period of four weeks. The biuret tests indicated that no "peptone" digestion had taken place in the sugar-containing tubes.

The behavior of *Bacillus coli* in the lactose media was about the same as in the glucose broth. In the tubes containing 0.2 per cent lactose the methyl red test suddenly changed from positive to negative. The indol test became positive with the decrease in hydrogen ion concentration. It appeared as if conditions gradually became favorable for growth, and that the rate of bacterial development increased as the acidity was neutralized. The coördination of the different tests is of special significance.

Fermentable sugar exerts the same influence on the metabolism of *Bacillus vulgaris* as on that of *Bacillus coli*. In the medium containing no carbohydrate the protein is completely broken down by the former organism, however. Within twenty-four hours there was appreciable indol formation. Indol was not produced, on the other hand, in any of the glucose-bouillon

tubes. The hydrogen ion concentration in these reached a point which inhibited the metabolic activities of the organism. This was apparent from the constancy of the biuret and the Sörensen figures. *Proteus vulgaris,* like *Bacillus coli,* was unable to ferment 0.4 per cent glucose completely, although 0.2 per cent was utilized in twenty-four hours.

Lactose was without effect on the protein metabolism of *Proteus vulgaris.* This was to be expected, of course, as this organism is generally regarded as being unable to attack lactose, in spite of a few claims to the contrary. The acidity, biuret and Sörensen figures, and the indol and sugar tests were the same in the lactose broth as in the plain peptone bouillon.

The above observations strongly indicate that the H ion concentration plays the important rôle in the inhibition of nitrogen metabolism in a medium containing a fermentable sugar. The failure of certain organisms to attack protein in the presence of carbohydrates is firmly established. That this failure is dependent on a coincident rise in the acidity of the medium is fully supported by the following experiments.

Media of the same composition as those already described were employed, with and without the addition of dipotassium phosphate. The media were made up as follows:

B	*2D*	*2DP*
Peptone, 0.5 per cent Beef extract, 0.25 per cent NaCl, 0.5 per cent	1 + 0.2 per cent glucose	2D plus 0.5 per cent K_2HPO_4
4D	*4DP*	*2L*
B plus 0.4 per cent glucose	4D plus 0.8 per cent K_2HPO_4	B plus 0.2 per cent lactose
2LP	*4L*	*4LP*
2L plus 0.5 per cent K_2PO_4	B plus 0.4 per cent lactose	4L plus 0.8 per cent K_2HPO_4

The dipotassium phosphate was used to serve as a buffer. The organisms employed were *Bacillus coli* and *Proteus vulgaris,*

both glucose and lactose media being inoculated with the colon bacillus, while the experiments with *Proteus vulgarus* were carried out in lactose broth alone, aside from the plain peptone broth cultures. Only the results showing the behavior of *Proteus vulgaris* in the glucose media are presented here in tabulated form (table 2), as they are thoroughly representative and well illustrate the influence of buffer action of the phosphate on the decomposition of the peptone in the presence of a fermentable sugar. With one exception, the results differ little from those obtained with *Bacillus coli* in both the glucose and lactose media, aside from the limited "peptone" utilization by *Bacillus coli* under even the most favorable conditions for protein metabolism.

The striking influence of the buffer reagent, dipotassium phosphate, on protein metabolism is most marked. The acidity of all of the sugar media increased during the first twenty-four hours. Subsequently the titratable acidity rapidly diminished in the solutions containing the phosphate, while in the other tubes it remained fairly constant. The biuret figures were in harmony with the Sörensen data. The reduction in the amounts of biuret-giving substances in the sugar media containing the phosphate was essentially the same as has been observed in plain peptone broth, the protein completely disappearing in two to three weeks. On the other hand, no reduction was noted in the sugar media to which no di-basic phosphate had been added. The Sörensen value of the phosphate tubes was increased two to three fold, while in the glucose broth, and in the case of *Bacillus coli* in lactose broth, containing no added phosphate, it remained practically constant.

The indol tests also indicated that protein metabolism occurred only in the solutions containing the buffer. In no instance was a positive indol test obtained in the glucose peptone broth to which no phosphate had been added. In both of the 0.2 per cent glucose media (with and without the buffer) *Bacillus coli* completely fermented the sugar, within twenty-four hours, while 0.4 per cent glucose was used up entirely only in the medium which contained the phosphate, from which it disappeared within the first twenty-four hours.

TABLE 2

Showing the influence of a buffer (K_2HPO_4) on the protein metabolism of Proteus vulgaris in a glucose medium

	REACTION				SÖRENSEN TEST				BIURET TEST				INDOL				SUGAR				METHYL RED			
	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP	2D	2DP	4D	4DP
Control.....	12.0	22.0	12.0	22.0	14.0	14.0	14.0	14.0	1.0	1.0	1.0	1.0	−	−	−	−	+	−	+	+	−	−	−	−
Inoculated																								
1 day..	28.0	36.0	30.0	34.0	16.0	14.0	16.0	10.0	1.0	1.0	1.0	1.0	−	+	−	−	+	+	+	+	+	−	+	−
3 days.	30.0	24.0	34.0	38.0	16.0	20.0	16.0	12.0	1.0	0.9	1.0	0.8	−	+	−	+	−	+	+	+	+	−	+	−
5 days.	30.0	28.0	30.0	34.0	16.0	24.0	14.0	18.0	1.0	0.9	1.0	0.9	−	+	−	+	−	+	+	+	+	−	+	−
7 days.	30.0	6.0	28.0	10.0	16.0	40.0	16.0	32.0	1.0	0.7	1.0	0.7	−	+	−	+	−	+	+	+	+	−	+	−
9 days	30.0	0.0	30.0	6.0	16.0	40.0	16.0	36.0	1.0	0.6	1.0	0.6	−	+	−	+	−	+	+	+	+	−	+	−
11 days.	28.0	*2.0*	30.0	*2.0*	18.0	42.0	16.0	42.0	1.0	0.5	1.0	0.35	−	+	−	+	−	+	+	+	+	−	+	−
13 days.	28.0	*2.0*	30.0	*2.0*	16.0	40.0	16.0	42.0	1.0	0.5	1.0	0.3	−	+	−	+	−	+	+	+	+	−	+	−
15 days.	30.0	8.0	26.0	*2.0*	16.0	22.0	16.0	42.0	1.0	0.7	1.0	0.15	−	+	−	+	−	+	+	+	+	−	+	−
17 days.	28.0	*4.0*	30.0	*4.0*	16.0	46.0	16.0	46.0	1.0	0.3	1.0	0.2	−	+	−	+	−	+	+	+	+	−	+	−
19 days.	28.0	*2.0*	30.0	*2.0*	18.0	44.0	18.0	46.0	1.0	0.2	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−
21 days.	30.0	*4.0*	32.0	*2.0*	16.0	40.0	16.0	42.0	1.0	0.0	1.0	0 0	−	+	−	+	−	+	+	+	+	−	+	−
23 days.	28.0	*4.0*	32.0	*6.0*	16.0	40.0	16.0	44.0	1.0	0.0	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−
25 days.	28.0	*2.0*	32.0	*4.0*	16.0	40.0	14.0	42.0	1.0	0.0	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−
27 days.	30.0	8.0	32.0	6.0	16.0	32.0	18.0	30.0	0.95	0.0	1.0	0.0	−	+	−	+	−	+	+	+	+	−	+	−

Italics indicate alkalin reaction to phenolphthalein.

Medium 2D
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.2 per cent

Medium 2DP
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.2 per cent
K_2HPO_4, 0.5 per cent

Medium 4D
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.4 per cent

Medium 4DP
Peptone, 0.5 per cent
Beef extract, 0.25 per cent
NaCl, 0.5 per cent
Glucose, 0.4 per cent
K_2HPO_4, 0.8 per cent

Proteus vulgaris manifested a peculiar behavior in the glucose tubes containing the di-basic phosphate, in which it failed to utilize the carbohydrate completely (see table 2). Even 0.2 per cent glucose was not completely fermented in the presence of the buffer. These results were indeed unexpected, and the tests were repeated, with identical results. It appears from these data that *Proteus vulgaris* readily attacks the nitrogen-containing substances in the absence of excessive acidity, and in preference to the residuary carbohydrate.

In all of the buffered media the methyl red test was negative, while in the sugar-containing solutions the hydrogen ion concentration was sufficiently increased within twenty-four hours to give the color reaction with methyl red.

GENERAL DISCUSSION

The influence of fermentable carbohydrates on nitrogen metabolism of bacteria is clearly shown in the foregoing experiments. The failure of certain organisms to attack protein or other complex nitrogenous foods in a medium containing carbohydrate is due to the accumulation of products which inhibit growth. The nitrogen which is required in the cell reproduction of the bacteria in the sugar medium is supplied by relatively simple substances, as for example amino acids and purin bases, which are always present in the ordinary media containing commercial peptone or meat extract, although in small amount.

The information furnished by the titratable acidity figures is of little value. Reactions which would ordinarily be considered too high apparently had no inhibitive action on continued bacterial development. The futility of adjusting the reaction to phenolphthalein was pointed out by Clark (1915) and further emphasized by Burton and Rettger (1917). It is now well known that culture fluids giving the same titration values may differ markedly in their hydrogen ion concentration. As was shown in the present work, a culture giving a methyl red negative test may have a titratable acidity twice as great as one that is methyl red positive.

The ability of *Bacillus subtilis* to break down protein in the presence of fermentable sugar, and in the absence of an added buffer, may be explained as follows. This organism attacks glucose slowly, and for this reason it is able to produce its proteolytic enzyme before the hydrogen ion concentration reaches a point unfavorable to further growth. When the enzyme is thus formed the products of the nitrogen metabolism neutralize the acid, at least in a measure, and the metabolism therefore continues uninterruptedly.

It is probable that the metabolism of *Bacillus coli* in a glucose or lactose medium in which much of the nitrogen is in a form directly available for utilization by the organism is such as to maintain a hydrogen ion concentration suitable for normal development without the aid of a buffer. In a case like this the nitrogen metabolism is rapid enough to neutralize the acid formed from the sugar decomposition. Such a condition has been shown in cultures of *Bacillus coli* in lactose media to which tryptophan was added (Distaso, 1913). Indol production could be demonstrated easily.

A recent experiment in which the metabolism of *Bacillus cloacae* was investigated clearly indicates that this organism behaves as *Bacills subtilis* does, except that the sugar is fermented more rapidly by the former. The final products of glucose and lactose fermentation by *Bacillus cloacae* are largely carbon dioxide and hydrogen, and hence the hydrogen ion concentration is not altered greatly.

The four organisms, *B. subtilis, B. cloacae, B. coli* and *P. vulgaris,* illustrate three distinct types of metabolism. *Bacillus subtilis* attacks protein in the presence of fermentable sugar, because the sugar is fermented slowly and the nitrogen and carbohydrate metabolism are maintained in equilibrium. *Bacillus cloacae* is also able to regulate its own environment, although the sugar is fermented very rapidly, but comparatively little acid occurs among the end products, and hence bacterial development and a nitrogen metabolism are not inhibited. *Bacillus coli* and *Proteus vulgaris* rapidly ferment glucose with the formation of large amounts of acid. Providing there is sufficient

sugar present, these organisms may produce enough acid in twenty-four hours to prevent further metabolism. The presence of a buffer, like dipotassium phosphate, tends to prevent such inhibition.

Kendall and Walker's conception that the presence of glucose delays the production of the proteolytic enzyme can not be accepted. A bacterial proteolytic enzyme, as a rule, is not produced within the first twenty-four hours, but requires a longer period before it makes its appearance in detectable quantities. Previous to enzyme formation some anabolism must take place, unless some enzyme has been transferred in the process of inoculation. In the tests in which the buffer reagent was employed the proteolytic enzyme appeared as soon in the sugar media as in the plain bouillon.

A positive indol test does not necessarily indicate that the sugar has been entirely fermented. Homer's explanation that the presence of glucose in a medium prevents indol formation because it forms a chemical combination with the tryptophan is therefore based on a false assumption.

The present investigation has shown conclusively that fermentable sugars in moderate amounts do not affect the nitrogen metabolism of bacteria, providing experimental conditions are favorably maintained, or in other words, under conditions of favorable environment. The common belief in a so-called "sparing action" of sugars in a protein medium is untenable, in the light of these experiments. According to this idea protein nitrogen is left unattacked, and thus spared from all participation in the metabolism. There is a true sparing action, however, in the sense that the nitrogen is utilized merely for growth, and that the sugar furnishes the energy. For example, the more luxuriant growth of the organisms here used in the glucose media containing the buffer as compared with plain peptone broth, may be explained on the basis that the nitrogen was used for growth, and that the sugar furnished the necessary energy supply. In plain peptone broth the nitrogenous food material furnishes both the energy and the nitrogen for necessary cell metabolism.

CONCLUSIONS

1. The presence of a carbohydrate in a culture medium may inhibit protein metabolism, depending on the nature of the medium and on the type of the organism.

2. The presence of sufficient buffer in a medium encourages continued normal nitrogen metabolism.

3. A utilizable carbohydrate in a medium has a true 'protein sparing' effect providing the hydrogen ion concentration is maintained within suitable limits.

REFERENCES

AUBEL, E., ET COLIN, H. 1913 Action des sucres sur la fonction pigmentaire du Bacille pyocyanique. Compt. rend. Soc. Biol., **75**, 25–7.

BAGINSKY, A. 1889 Zur Biologie der normalen Milchkothbakterien. Zeitschr. physiol. Chem., **13**, 352–64.

BENEDICT, S. R. 1908–09 A reagent for the detection of reducing sugars. Jour. Biol. Chem., **5**, 485–7.

CLARK, W. M. 1915 The reaction of bacteriologic culture media. Jour. Inf. Dis., **17**, 109–36.

DE GRAAF, W. C. 1909 Untersuchungen über Indolbildung der Bacterium coli commune. Centralbl. Bakt., Abt. I, Orig., **49**, 175–8.

DISTASO, A. 1913 Sur la production de l'indol par le B. coli en milieux au tryptophane et sucres. Compt. rend. Soc. Biol., **75**, 200–1.

FISCHER, A. 1915 Hemmung der Indolbildung bei Bacterium coli in Kulturen mit Zuckerzusatz. Biochem. Zeitschr., **70**, 105–18.

GLENN, T. H. 1911 Variation and carbohydrate metabolism of bacilli of the Proteus group. Centralbl. Bakt., Abt. I, Orig., **58**, 481–95.

HOMER, A. 1916 A suggestion as to the cause of the lessened production of indol in media containing glucose. Jour. Hyg., **15**, 401–4.

JONES, H. M. 1916 The protein-sparing action of utilizable carbohydrates in cultures of certain sugar-fermenting organisms. Jour. Inf. Dis., **19**, 33–45.

KENDALL, A. I., DAY, A. A. AND WALKER, A. W. 1913 Observations on the relative constancy of ammonia production by certain bacteria. Jour. Inf. Dis., **13**, 425–8.

KENDALL, A. I., AND WALKER, A. W. 1915 Observations on the proteolytic enzyme of Bacillus proteus. Jour. Inf. Dis., **17**, 442–53.

KLIGLER, I. J. 1915 Observations on the metabolism of Bacillus vulgaris. Biochem. Bull., **4**, 195–6.

LIBORIUS, P. 1886 Beiträge zur Kentniss des Sauerstoffbedürfnisses der Bakterien. Zeitschr. Hyg., **1**, 115–76.

PÉRÉ, M. A. 1892 Contribution a la biologie du Bacterium coli commune et du Bacille typhique. Annal. de l'Inst. Pasteur, **6**, 512–37.

Rougentzoff, D. 1913 La fermentation de divers sucres par le B. coli et la production de l'indol. Compt. rend. Soc. Biol., **74**, 1098–1100.

Sears, H. J. 1916 Studies in the nitrogen metabolism of bacteria. Jour. Inf. Dis., **19**, 105–37.

Sörensen, S. P. L. 1908 Enzymstudien. Biochem. Zeitschr. **7**, 45–101.

Vernon, H. M. 1904 The peptone-splitting ferments of the pancreas and intestine. Jour. physiol., **30**, 330–69.

Winternitz, H. 1892 Ueber das Verhalten der Milch und ihres wichtigsten Bestandtheile bei der Fäulniss. Zeitschr. physiol. Chem., **16**, 460–87.

STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

VIII. THE SUBGROUPS AND GENERA OF THE ACTINOMYCETALES

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Order III. **Actinomycetales.** *Nom. Nov.*

Synonyms:

Actinomycetes Balbiani, 1886, p. 542.
Trichobacteriacei Fischer, 1895, p. 138 in part.

Mold-like organisms, not typically water forms, saprophytic or parasitic. Sheath not impregnated with iron, true hyphae with branching often evident, conidia may be developed, but never endospores. Without granules of free sulphur and without bacteriopurpurin. Never producing a pseudoplasmodium. Always nonmotile.

The order *Actinomycetales* contains a single family, *Actinomycetaceae.*

Family I. **Actinomycetaceae.** Fam. nov.

Characters same as those of the order.

The following names have been used for genera which may be included in this family.

Actinobacillus Brumpt, 1900, p. 849
Actinobacterium Haass, 1906, p. 180
Actinocladothrix Affanassieff, 1888, p. 79
Actinomyces Harz, 1877, p. 125
Cohnistreptothrix Pinoy, 1911
Viscomyces Rivolta and Micellone, 1879, p. 145
Leptotrichia Trevisan, 1879, p. 138

Micromyces Gruber, 1891, p. 648
not *Micromyces* Dangeard, 1888, p. 55
Nocardia Trevisan, 1889, p. 9
Streptothrix Cohn, 1875, p. 186
not Corda, 1839
Thermoactinomyces Tsilinsky, 1899, p. 500
Rasmussenia De Toni and Trevisan, 1889, p. 930

The genus *Actinobacterium* has had no definite specific names ascribed to it.

The following are invalid because previously used for other distinct groups of organisms: *Micromyces* and *Streptothrix*.

The name *Actinocladothrix* was used by Affanassieff in the combination *Bacterium actinocladothrix*, but several authors have listed the name as though it had been used as a genus.

The following generic names are therefore to be considered, or at least are not invalid for any of the preceding reason: *Actinobacillus*, *Actinomyces*, *Cohnistreptothrix*, *Discomyces*, *Leptotrichia*, *Nocardia*, *Thermoactinomyces*, *Rasmussenia*.

The genera may be differentiated by the following key.

Key to the genera of Actinomycetaceae

A. No evident *aerial* threads or conidia formed. Usually parasitic. Often anaërobic or microaërophilic.
  1. Threads usually not branched.
    a. Threads disjointing very readily; long mycelial threads uncommon. Genus 1. *Actinobacillus*
    b. Threads longer, not disjointing into short rods. Genus 2. *Leptotrichia*
  2. Threads more or less branched, frequently clubbed in tissues. Genus 3. *Actinomyces*

B. Aërial threads and conidia evident on culture media.....Genus 4. *Nocardia*

Genus 1. **Actinobacillus** Brumpt, 1900, p. 849

Filament formation; resembling streptobacilli. In lesions no mycelium formed, but at peripheries finger shaped branched cells are visible. Gram negative. Not acid fast.

Possibly the genus belongs with the *Bacteriaceae*. It is evidently a transition form.

The type species is *Actinobacillus lignieresi* Brumpt, the cause of actinobacillosis in cattle.

Genus 2. **Leptotrichia** Trevisan, 1879, p. 138

Synonyms:
Leptothrix Robin, 1847, p. 345
not *Leptothrix* Kuetzing, 1843, p. 198
Rasmussenia Trevisan, 1889, p. 930

Rod shaped or filamentous cells, non motile, unbranched, without aërial hyphae or conidia; parasites or facultative parasites.

The type species is *Leptotrichia buccalis* (Robin) Trevisan. This genus is commonly termed *Leptothrix*, but certainly forms as unlike as *Leptothrix ochracea* and *Leptotrichia buccalis* do not belong in the same genus. *Leptotrichia* was created by Trevisan in 1879 with *L. buccalis* as the only species, but in 1889 he enlarged the genus, removing the mouth forms to the genus, *Rasmussenia*.

Genus 3. **Actinomyces** Harz, 1877, p. 125

Synonyms:
Streptothrix Cohn, 1875, p. 186
not *Streptothrix* Corda
Discomyces Rivolta and Micellone, 1879, p. 145
Micromyces Gruber, 1891, p. 648
not *Micromyces* Dangeard, 1888, p. 55
Nocardia Trevisan (in part)
Oospora Sauvageau and Radais, 1892, p. 242
not *Oöspora* Wallroth, 1833, p. 182
Cohnistreptothrix Pinoy, 1911

Branched filaments, resembling mycelium, breaking up into segments which may function as conidia. Usually parasitic. Clubbed ends conspicuous in lesions. Not producing aërial hyphae or conidia.

The type species is *Actinomyces bovis* Harz, the cause of bovine actinomycosis.

Genus 4. **Nocardia** Trevisan, 1889, p. 9

Synonyms:

Actinomyces of many authors
Streptothrix of many authors
Thermoactinomyces Tsilinsky, 1899, p. 500

Branched filaments, resembling a mycelium, readily breaking up into segments. Usually saprophytic. Aërial threads and conidia commonly produced.

REFERENCES

AFFANASSIEFF, M. J. 1888 Ueber die klinische Mikroskopie und Bakteriologie der Aktinomycosis. St. Petersburger med. Wochenschrift. New Series, **5**, 79.

BRUMPT, E. 1910 Precis de Parasitologie. Paris.

COHN, FERDINAND 1875 Untersuchungen über Bakterien II. Beiträge z. Biologie d. Pflanzen, **1** (Heft. 3) 141–208.

DANGEARD, A. 1888 Memoire sur les Chytridinees. Le Botaniste, **1**, 55.

DE TONI AND TREVISAN 1889 Schizomycetaceae. Naegeli. In Saccardo's Sylloge Fungorum, **8**, 923–1087.

FISCHER, ALFRED 1895 Untersuchungen über Bakterien. Jahresber. f. wiss. Bot., **27**, 1–163.

GRUBER, M. 1891 Micromyces Hoffmanni. Cent. f. Bakt. Par. u. Inf., **10**, 648.

HAASS, EVERHARD 1906 Beiträge zur Kenntniss der Aktinomyceten. Cent. f. Bakt. Par. u. Inf. Abt. 1, **40**, 180–186.

HARZ, C. O. 1877–78 *Actinomyces bovis*, ein neuer Schimmel in dem Gewebe des Rindes. Jahresber. d. Muenchener Central Thierarznei Schule, 1877–78, p. 125–140.

KÜTZING, F. T. 1843 Phycologia generalis,

LACHNER-SANDOVAL, V. 1898 Ueber Strahlenpilze. Inaugural-diss. Strassburg.

LEHMANN AND NEUMANN 1896 Altas und Grundriss der Bakteriologie.

PINOY, E. AND MORAX 1911 Sur les concretions des voies lacrymales. Etude mycologique Bull. soc. Ophtalmologie de Paris, no. 3.

RIVOLTA AND MICELLONE 1882 Giornale Guglielmo da Saliceto, Piacenza. p. 145. 1879. Giornale di Anat. e. Fis. degli. An. Pisa.

ROBIN 1853 Historie naturelle des vegetaux parasites.

SAUVAGEAU, C. AND RADAIS, M. 1892 Sur les genres Cladothrix, Streptothrix, Actinomyces et description de deux Streptothrix nouveaux. Ann. d. l'Inst. Pasteur **1**, 242–273.

TREVISAN, V. 1879 Prime linee d'introduzione allo studio dei Batterj. italiani. Rendiconti. Reale Istituto Lombardo di Scienze e Lettere IV. Series 2, **12**, 133–151.

TREVISAN, V. 1889 Gen. e spec. delle Bacteriaceae.

TSILINSKY, P. 1899 Sur les Mucidineés Themophiles Ann. de l'Inst. Pasteur, **13**, 500.

WALLROTH 1833 Flora crypt. germ., **2**, 182.

THE CLASSIFICATION OF THE ACIDURIC BACTERIA

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The proliferation of the aciduric bacteria apparently depends upon the almost exclusive utilization of carbohydrates or carbohydrate-like substances. The corollary of this property, that is, the ability to survive in the presence of considerable amounts of acid is made use of in their isolation. The titles applied to this group have varied with the authors who have studied it. The term "Lactic acid bacteria" is certainly too broad, since its use would force the inclusion of organisms that are of an entirely different type, such as *B. coli*. "Bulgaricus" and "Caucasicum" are unsuitable terms because these bacilli are neither the most frequently occurring nor the typical members of this group. "Acidophilus," while more nearly appropriate, is still inexact, since this bacillus is characterized by its acid resisting rather than by its acid "loving" properties. Upon the whole, the word "aciduric," adopted by Kendall (1910) appears to be the most fitting term under which to group these organisms.[1]

The distribution of the aciduric bacteria calls for no comment, their ubiquity as a group has long been known. The distribution of individual members, however, is less well understood. In 1909 Heinemann and Hefferan published a paper in which it was stated that *B. acidophilus* and *B. bulgaricus* were identical. It seemed to the writer that much of the work on this subject, both preceding and following that of these authors, was not as convincing as might be, owing to the failure of practically all

[1] Distaso (Cent. f. Bakt. Orig. 1911, **59**, 48) employed the term acid-tolerant.

workers to provide a suitable medium for the cultivation of these bacteria.

In 1914 Rahe was able to show that these organisms would develop with a very satisfactory degree of luxuriance in unneutralized meat-peptone-broth containing a suitable carbohydrate. We have carried various strains of aciduric organisms for years in this broth. Although the writer has in subsequent communications tried to place sufficient emphasis on this fact, the fallacy still persists that these bacteria are not readily grown on the "usual" laboratory media.

Once the difficulty attending the cultivation of the aciduric bacteria was removed, an examination of the cultural characteristics of a number of strains brought to light several significant differences between the members of this group. It became evident that the Bulgarian bacillus could not utilize maltose (some strains could not ferment sucrose) and in this respect differed from the great majority of aciduric bacilli found in the human intestine and elsewhere. The latter could be conveniently grouped according to whether they clotted milk or failed to do so. The failure of *B. bulgaricus* to ferment maltose made it possible for the writer (1915) to demonstrate that this organism apparently does not exist as an intestinal inhabitant in human beings. The examination of hundreds of strains of aciduric bacilli from human feces that did not embody purposely ingested *B. bulgaricus* has failed to reveal a single organism of that type.

Rogers and Davis (1912) quote Hastings (1908), Hastings and Hammer (1909) and Heinemann and Hefferan (1909) as having shown that the Bulgarian bacillus is "widely distributed and may be isolated from almost any sample of milk." Most of these authors admit the difficulty of cultivating aciduric bacteria in glucose broth, and this is true if the "natural" acidity of the broth is interfered with. The unneutralized broth mentioned above overcomes this difficulty, probably owing to its unaltered amino acids or other growth-promoting substances, such as were described for the meningococcus by Lloyd (1917). In the present investigation organisms corresponding to the

Bacillus bulgaricus were isolated from milk in but two instances, fourteen other strains proving to be aciduric organisms of another type.

The *Bacillus acidophil-aerogenes* of Torrey and Rahe (1915) is an aciduric organism differing from the bacillus of Moro in that it forms gas. The *Bacillus bifidus* is easily identified upon morphological grounds. Opinion as to its anaerobic requirements has lately undergone modification (Howe, 1917). We know that the characters just mentioned differentiate the organisms in some fashion, but when as groups, and when as individual bacilli, has never been determined. The object of the present investigation is to determine whether a better classification might not be made.

The methods employed in this work were, with some modification, those used by the writer in his earlier investigations in this field. The selective medium was acetic acid-glucose-broth having an acidity of N/20. This was seeded with the material under examination and incubated for three days. The method of triple seeding employed by Kendall (1910) was early abandoned, confirmation of the aciduric nature of the organisms being obtained by primary cultivation in this broth of the colonies fished from agar plates. Unneutralized glucose-oleate agar was superseded by the glucose liver agar of Torrey (1917). This medium has a reaction of plus 3.

In the cultivation of the bacteria of the type of *B. acidophilus* incubation for two or three days is desirable, though this is not always necessary. All cultures were carried in the unneutralized meat-peptone-glucose broth, and, with the various test substances substituted for glucose, this broth was used in the cultural tests. The *Bacillus bifidus* was grown in 0.5 per cent glucose agar having an acidity of plus 2. The strains of this organisms were isolated in part from human and in part from canine stools. The new differential plating method devised by Torrey (1917) was employed for this purpose.

It will be seen upon reference to the table that the cultures examined were obtained from a variety of sources. In saliva the *Bacillus acidophilus* and the *Bacillus acidophil-aerogenes*

occurred with equal frequency, while the former bacillus was not so frequently encountered in house sewage or milk. In human and animal feces as well as in saliva there is at times a tendency towards the appearance of one species to the exclusion of the other. Stomach contents from five cases of gastric carcinoma were examined and both types of organisms isolated, thus disposing of the Boas-Oppler bacillus as a separate entity. None of the carcinoma strains corresponded to *B. bulgaricus*, differing in this from some strains isolated by Heinemann (1917).

The classification herein suggested is based on fermentation tests. Among the aciduric bacilli, other than *B. bifidus*, colony formation, morphology and staining properties are subject to variation common to all. In the case of *B. bulgaricus*, action on milk and possibly colony formation assume the importance of distinct characters.

BACILLUS ACIDOPHILUS

As table 1 shows, it would be possible, on the basis of fermentation tests to define six subtypes of *B. acidophilus*. Mannite proved both here and elsewhere to be a very unsatisfactory test substance, permitting a very meager development at best. If we regard this alcohol as without value in differentiation the number of types becomes reduced to four. It is evident that the great majority of these organisms were milk clotters, thus agreeing with the *B. acidophilus* of Moro. The organism of Finkelstein does not possess this property. I am unable to account for the rarity of the non-clotting bacillus in this instance. In a previous investigation it was encountered with much greater frequency (Rahe 1914).

B. ACIDOPHIL-AEROGENES

The variable behavior of this organism in milk was noticed in the paper in which it was originally described (Torrey and Rahe 1915). The fermentation tests show a greater unity here than was the case in the preceding group. Sixty-five per cent of the cultures produced gas in all of the test substances if we except

TABLE 1

Non-gas producing aciduric bacilli (*B. acidophilus*)

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK-TIME OF CLOTTING	INCREASE ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHILUS
Sa- 7	Saliva	I	Good	24 hours			
Sa-18	Saliva	I	Good	24 hours	11.7	A_1Fermented	
Sa-23	Saliva	I	Heavy	48 hours	4.2	Glucose	
Sa-24	Saliva	I	Heavy	48 hours	7.1	Maltose	
Sa-25	Saliva	I	Heavy	18 hours	11.7	Raffinose	
Sa-26	Saliva	I	Heavy	24 hours	12.3	Sucrose	
Sa-27	Saliva	I	Heavy	48 hours	7.0	Lactose	
Sa-22	Saliva	I	Good	48 hours	12.2	Mannite	
506	Feces	I	Heavy	24 hours	7.3		
G	Feces	I	Heavy	24 hours			
D G C	Feces	I	Heavy	24 hours			
M-1	Milk	I	Good	18 hours			
M-4	Milk	I	Fair	18 hours			A
S-1	Carcinoma	I	Good	48 hours	7.2		
S-8	Carcinoma	I	Good	48 hours	13.7		
G-2	Feces	I	Good	Not clotted	7.9		
Har	Feces	I	Good	Not clotted	4.6	A_2Fermented	
Sa-3	Saliva	I	Good	24 hours		Glucose	
Sa-6	Saliva	I	Fair	48 hours		Maltose	
Sa-5	Saliva	I	Heavy	48 hours		Raffinose	
Sa-10	Saliva	I	Fair	48 hours		Sucrose	
515	Dog feces	I	Good	18 hours	11.3	Lactose	
D	Feces	I	Good	Not clotted	6.6		
M 11	Milk	I	Heavy	18 hours	8.7		
Sa-21	Saliva	I	Heavy	48 hours	12.6	B_1Fermented	
						Glucose	
507	Feces	I	Heavy	24 hours	8.0	Maltose	
K	Feces	I	Heavy	24 hours	12.2	Sucrose	
M 10	Milk	I	Heavy	18 hours	11.1	Lactose	
S_2	Carcinoma	I	Heavy	5 days	7.2	Mannite	B
Ber. C	Feces	I	Heavy	48 hours		B_2Fermented	
Sa-1	Saliva	I	Good	48 hours		Glucose	
Sa-2	Saliva	I	Good	72 hours		Maltose	
Sa-11	Saliva	I	Fair	24 hours		Sucrose	
502	Sewage	I	Good	6 days	13.0	Lactose	
M 5	Milk	II	Good	18 hours			
						Fermented	
						Glucose	C
1-a	Feces	I	Good	Not clotted		Maltose	
B	Feces	I	Good	Not clotted		Sucrose	
Sa-6	Saliva	I	Fair	48 hours		Fermented	
						Glucose	D
						Maltose	

TABLE 2

B. acidophil-aerogenes

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK-TIME OF CLOTTING	INCREASE IN ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHIL-AEROGENES
Sa-19	Saliva	I	Heavy	48 hours	8.3	Fermented (gas)	
Sa-20	Saliva	I	Heavy	7 days	10.3	Glucose	
H-1	Feces (Hen)	I	Heavy	15 days		Maltose	
Sa-32	Saliva	I	Heavy	72 hours	9.3	Raffinose	
Sa-33	Saliva	I	Heavy	72 hours	11.3	Sucrose	
Sa-34	Saliva	I	Good	72 hours	8.0	Lactose	
Sa-35	Saliva	I	Good	24 hours	7.7		
Sa-36	Saliva	I	Good	5 days	13.1		
Berd	Feces	I	Fair	8 days			
501	Sewage	I	Good	10 days	11.5		
511	Sewage	I	Heavy	48 hours	11.2		
F	Feces	I	Heavy	Not clotted			
C-2	Feces	I	Heavy	Not clotted	8.0		
Baby 1	Feces	I	Heavy	Not clotted	12.0		
Baby 2	Feces	I	Heavy	Not clotted	13.5		
Baby 3	Feces	I	Heavy	Not clotted	12.5		
Cs 4	Feces	I	Heavy	Not clotted	8.0		
Jam	Feces	I	Heavy	10 days	10.5		
Buck	Feces	I	Heavy	72 hours	9.5		
C-6	Feces	I	Heavy	Not clotted	8.0		
C-5	Feces	I	Heavy	Not clotted	8.5		
H	Feces	I	Heavy	Not clotted	10.5		A
C-1	Feces	I	Heavy	Not clotted	9.0		
Case 3	Feces	I	Heavy	Not clotted	9.3		
Bel-9	Feces	I	Heavy	7 days			
X-1	Feces (hen)	I	Heavy	21 days			
D-7a	Feces (dog)	II	Heavy	18 hrs.			
D-7b	Feces (dog)	I	Heavy	9 days			
D-8a	Feces (dog)	II	Heavy	8 days	5.2		
D-8b	Feces (dog)	II	Fair	8 days	6.3		
D-8c	Feces (dog)	II	Heavy	7 days	6.2		
R-8	Stomach carcinoma	II	Heavy	48 hours	4.5		
R-13	Stomach carcinoma	I	Heavy	7 days	6.8		
L-1	Stomach carcinoma	I	Heavy	15 hours	14.5		
S-2	Stomach carcinoma	II	Heavy	18 hours	7.4		
Ro-1	Stomach carcinoma	I	Heavy	Not clotted	8.3		
L-3	Stomach carcinoma	I	Heavy	Not clotted	8.0		
505	Sewage	I	Heavy	6 days	7.3	Growth without gas in raffinose	B
509	Feces (monkey)	II	Heavy	48 hours	8.3		

TABLE 2—*Continued*

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK-TIME OF CLOTTING	INCREASE IN ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHIL-AEROGENES
C-3	Feces	I	Heavy	Not clotted		Gas in Glucose Maltose Sucrose Lactose	B
D-1	Feces (dog)	I	Heavy	15 days			
M-12	Milk	II	Good	6 days	4.9		
M-16	Milk	II	Good	18 hours	11.1		
S-7	Stomach carcinoma	I	Good	48 hours			
M-14	Milk	II	Good	12 days	5.0	No growth in raffinose Gas in Glucose Maltose Sucrose Lactose	C
M-15	Milk	II	Good	6 days	4.0		
Sa-3	Saliva	I	Fair	24 hours		Growth without gas in lactose and raffinose Gas in Glucose Maltose Sucrose	D
503	Sewage	I	Heavy	Not clotted	10.0		
M-17	Milk	II	Good	8 days			
Sa-16	Saliva	I	Good	Not clotted	6.5	No growth in lactose and raffinose Gas in Glucose Maltose Sucrose	E
504	Sewage	I	Heavy	Not clotted	10.0		
Sa-15	Saliva	I	Fair	48 hours	9.0	No growth in lactose, raffinose and sucrose Gas in Glucose Maltose	F
Sa-28	Saliva	I	Good	Not clotted	8.2	Growth without gas in lactose Gas in Glucose Maltose Raffinose Sucrose	G
Sa-29	Saliva	II	Good	Not clotted	6.4		
500	Sewage	I	Good	14 days	6.5		
M-2	Milk	I	Good	18 hours			
D-3	Feces (dog)	I	Heavy	48 hours			

TABLE 2—*Concluded*

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UNNEUTRALIZED GLUCOSE BROTH	MILK—TIME OF CLOTTING	INCREASE IN ACIDITY GLUCOSE BROTH 5 DAYS	CULTURAL	B. ACIDOPHIL-AEROGENES
Ro. 1	Stomach carcinoma	I	Good	12 days	4.7	Growth without gas in sucrose Gas in Glucose Maltose Raffinose Lactose	H
508	Feces (monkey)	I	Good	Not clotted	4.7		

mannite. The disparity in numbers between the bacillus A and the next most numerous member of this group hints that we may be dealing with a main type and its variants. Plausibility is given to this suspicion by the progressive nature of the assumed lapses in character. The diagram below will illustrate this point.

B. acidophil-aerogenes *A*
(Gas in all test-substances)

B. acidophil-aerogenes *G*
(No gas in lactose)

B. acidophil-aerogenes *H*
(No gas in sucrose)

B. acidophil-aerogenes *B*
(No gas in raffinose)

B. acidophil-aerogenes *C*
(No growth in raffinose)

B acidophil-aerogenes *D*
(No gas raffinose and lactose)

B. acidophil-aerogenes *E*
(No growth in raffinose and lactose)

B. acidophil-aerogenes *F*
(No growth in raffinose, lactose or sucrose)

The diagram represents observed "lapses" only. More extended observations are necessary if the sequence is to be correct. There might be found, for example, the bacillus intermediate between *E* and *F*; i.e., one that grows in sucrose but does not produce gas and does not grow in raffinose and lactose.

Although the failure of *B. acidophil-aerogenes* first, to produce gas in a given carbohydrate and then to ferment it entirely, is suggestive in view of the numerical relationships of the different strains, we are not at liberty to conclude absolutely that such failures are instances of suppressed function and not real cultural differences. The cultural differences shown in the table are those found in freshly isolated strains and were not brought about intentionally. Prolonged artificial cultivation or other means might bring about such alterations, but until these are observed actually to occur it is better to assume that these differences are absolute and classify the organisms accordingly.

BACILLUS BULGARICUS

In table 3 are the organisms that correspond to the *Bacillus bulgaricus*. Some of these strains were obtained from the American Museum of Natural History while others were from commercial preparations of one kind or another. One strain was isolated from the saliva of a man, who, as far as could be determined, had not recently taken this bacillus. Two strains came from grade B. milk and made it appear that *B. bulgaricus*, in common with other lactic acid bacteria is able to survive the temperature of pasteurization. Experiments made to test the heat resisting properties of *B. acidophilus*, *B. acidophil-aerogenes* and *B. bulgaricus* showed them to be capable, in twenty-four hour broth culture, of surviving moist heat for one hour at 65°C.

Examination of the table shows that there is a very fair degree of uniformity between the various members of this group, both as regards colony formation and action on milk, fifteen out of seventeen strains showing close resemblance in this respect. Cultural tests permit their separation into four types. White and Avery (1910) recognized two types of *B. bulgaricus*. Their type A consisted of bacilli that were rapid fermenters, forming inactive lactic acid and exhibiting no granules, while type B showed granules, was less active and formed levo-rotary acid. These characteristics are not exclusive properties of *B. bulgaricus*.

TABLE 3

B. bulgaricus

CULTURE	SOURCE	COLONY TYPE	GROWTH IN UN-NEUTRALIZED GLUCOSE BROTH	MILK, TIME OF CLOTTING	MILK, ACIDITY IN 6 DAYS	CULTURAL	
1	Museum	II	Good	18 hours	26.3	Fermented	
2	Fairchild	II	Good	18 hours	25.3	Glucose and Lactose	
3	Museum	II	Good	24 hours	26.5		
5	Museum	II	Good	18 hours	20.9		
6	Fermented milk	II	Good	18 hours	13.0		
7	Fermented milk	II	Good	24 hours	14.8		A
8	Fermented milk	II	Good	24 hours	14.4		
9	B. B.	II	Good	24 hours	25.4		
11	Tablet	II	Good	24 hours	28.0		
M 7	Grade B milk	II	Good	18 hours	25.0	Fermented	
M 8	Grade B milk	II	Good	18 hours	26.0	Glucose, Lactose and Raffinose	B
B^1	B. B.	II	Good	48 hours	24.0		
B_2	Fairchild	II	Good	18 hours	26.0		
10	Massolin	II	Good	24 hours	11.4	Fermented	
12	Tablet	II	Good	24 hours	24.6	Glucose, Lactose	C
Sa8	Saliva	I	Good	48 hours		and Sucrose	
4	Museum	II		18 hours	25.7	Fermented Glucose, Lactose, Sucrose and Raffinose	D

BACILLUS BIFIDUS

This organism is usually classed with the aciduric bacteria, presumably because of its occurrence in infant feces. That the bacillus is truly aciduric was shown by tests in which various strains were grown anaerobically in N/20 acetic-acid-glucose-

broth, with and without the addition of blood. Grown in unneutralized glucose broth, some strains produced an acidity of plus seven after five days incubation. I was unable to detect gas even when blood was added to the medium. Anaerobic milk cultures gave irregular results. Some cultures clotted the milk in periods ranging from eight to fifteen days, giving rise to an acidity that did not go beyond plus 7. In other instances the organisms failed to grow.

The figures in the table below represent the increase in acidity in puncture cultures in 0.5 per cent agar containing 1 per cent of the various test substances. The incubation period was five days.

TABLE 4

B. bifidus. Increase in acidity in 0.5 per cent agar after five days at 37°

CULTURE	SOURCE	MALTOSE	SUCROSE	LACTOSE	RAFFINOSE	GLUCOSE	MANNITE
D-7	Dog feces	3.3	6.0	4.1	3.4	3.7	0.0
D-1	Dog feces	7.1	5.0	2.4	4.6	2.4	0.0
C.	Human feces	4.0	2.1	3.2	2.9	3.4	0.0
D-3	Dog feces	0.8	0.7	1.9	1.9	2.5	0.0
D-9	Dog feces	3.6	2.6	5.4	1.2	6.6	0.0
D-6	Dog feces	3.5	1.8	3.1	2.6	3.6	0.4
Saun	Human feces	5.1	3.4	5.3	4.9	4.0	0.0
Pup	Puppy feces	13.3	5.0	11.9	9.6	9.8	2.2
Nor.	Human feces	5.7	2.5	4.6	5.1	4.0	0.5
D-11	Dog feces	8.3	6.4	9.0	9.3	9.8	2.4
D-1a	Dog feces	11.1	8.3	12.2	10.1	9.1	1.1
D-9a	Dog feces	+	+	+	+	+	+
D-9b	Dog feces	+	+	+	+	+	+
McE.	Human feces	+	+	+	+	+	+

In the latter part of this experiment the process of sugar freeing the broth used in the preparation of the medium was carried beyond the usual stage to determine whether the products resulting from the greater development of *B. coli* served to enrich the agar. Table 5 shows the results of comparative tests of half per cent agar, one lot of which was subjected to more extensive proteolysis by *B. coli* than the other. A distinct cloudiness accompanied the increased acid production. It is evident that the earlier cessation of growth in some lots of medium is due,

not to the accumulation of acid products of growth, but rather to the exhaustion of essential amino acids or other growth promoting substances.

Aside from their action on mannite and milk all of the strains of *B. bifidus* acted uniformly, and if we limit ourselves to the easily applied cultural tests in 0.5 per cent agar we find but two possible types of this organism. Even that arrangement would require defense, since the action on mannite was always slight. While the nature of *B. bifidus* is not yet entirely settled, Noguchi's (1910) demonstration of its pleobiosis has lately been confirmed

TABLE 5

Showing the greater activity of B. bifidus in medium 2, prepared from broth which had previously been subjected to the more prolonged action of B. coli

CULTURE	SOURCE	MALTOSE	SUCROSE	LACTOSE	RAFFINOSE	GLUCOSE	MANNITE	MEDIUM LOT
Pup	Puppy feces	2.8	3.4	3.1	2.6	3.7	0.5	1
Pup	Puppy feces	13.3	5.0	11.9	9.6	9.8	2.2	2
D 11	Dog feces	4.6	3.1	3.6	3.4	5.8	0.7	1
D 11	Dog feces	8.3	6.4	9.0	9.3	9.8	2.4	2
D 1a	Dog feces	2.9	1.6	4.5	0.4	4.9	0.4	1
D 1a	Dog feces	11.1	8.3	12.2	10.1	9.1	1.1	2

by Howe (1917), and in view of its protean nature, we must keep in mind that we are here dealing with the aciduric phase of this organism only. In its aciduric phase, then, the *Bacillus bifidus* is a non-gasforming organism that may or may not clot milk but ferments maltose, glucose, lactose, sucrose and raffinose.

The figures shown on opposite page illustrate the relative frequency with which the individual members of each of three of the groups of aciduric bacilli were found during the course of the present investigation.

It is unfortunate that, except in case of *B. bulgaricus*, the action on milk could not be correlated with the other cul-

tural test. In the instance of organisms of the type of *B. acidophilus* 90 per cent of the strains clotted milk, while with *B. acidophil-aerogenes* this figure falls to 65.

There is, in recent bacteriological literature, an instance illustrative of the desirability for a definite classification of the aciduric

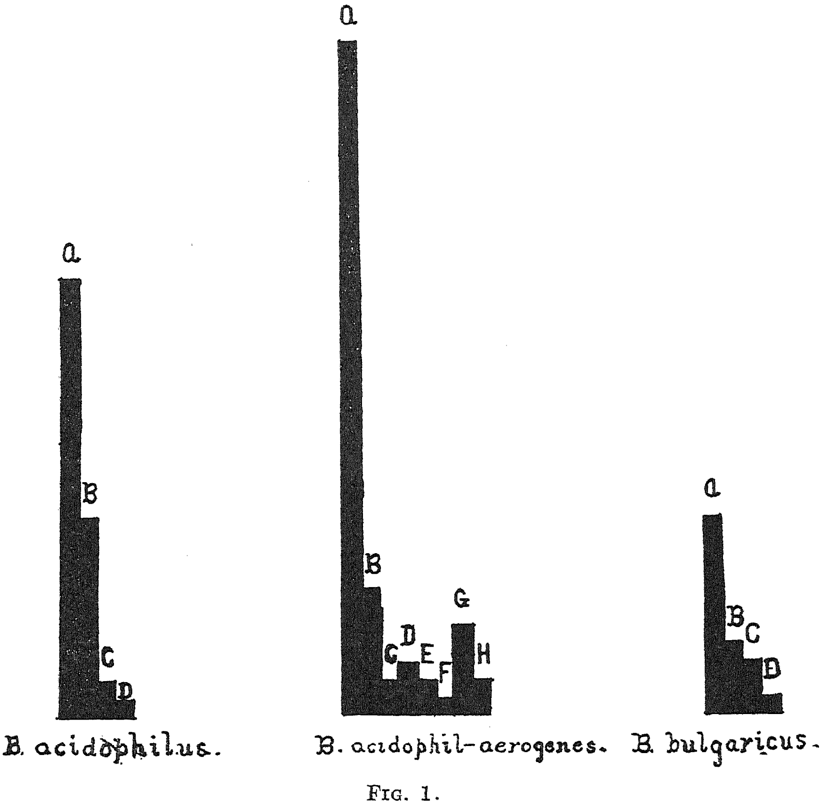

FIG. 1.

bacteria. Clark (1916), was unable to come to a definite conclusion as to the H ion concentration of the *Bacillus bulgaricus* owing in part to his uncertainty as to the identity of the particular organisms with which he was dealing. A classification, such as is given in the table below, would simplify investigations involving aciduric bacilli.

TABLE 6

Scheme for the classification of the aciduric bacilli including the aciduric phase of B. bifidus

BACILLUS		MALTOSE	GLUCOSE	LACTOSE	SUCROSE	RAFFINOSE	MILK
B. acidophilus	A	+	+	+	+	+	±
	B	+	+	+	+	−	±
	C	+	+	−	+	−	−?
	D	+	+	−	−	−	±
B. acidophil-aerogenes	A	g	g	g	g	g	±
	B	g	g	g	g	+	±
	C	g	g	g	g	−	±
	D	g	g	+	g	+	±
	E	g	g	−	g	−	−?
	F	g	g	−	−	−	+?
	G	g	g	+	g	g	±
	H	g	g	g	+	g	±
B. bulgaricus	A	−	+	+	−	−	+
	B	−	+	+	−	+	+
	C	−	+	+	+	−	+
	D	−	+	+	+	+	+
B. bifidus Aciduric phase		+	+	+	+	+	±

+ = fermentation of carbohydrate or clotting of milk.
G = gas formation.
± = positive reaction in some instances negative in others.
− = no growth.

CONCLUSION

The aciduric bacilli, including *B. bifidus* in its aciduric phase, may best be classified in accordance with their abilities to ferment certain carbohydrates.

REFERENCES

Clark, W. M. 1916 The acid production of *B. bulgaricus*. Proc. Soc. Am. Bact. 59.

Howe, P. R. 1917 Microorganisms of dental caries. J. Med. Research, **31**, 481.

Hastings, E. G. 1908 A preliminary note on a group of lactic acid bacteria not previously described in America. Science, **28**, 656.

HASTINGS, E. G., AND HAMMER, B. W. 1909 The occurrence and distribution of lactic acid organisms resembling the bacillus bulgaricus of yogurt. Wisconsin Agricultural Experiment Station Research Bulletin 6, 197.

HEINEMANN, P. G. AND HEFFERAN, M. 1909 A study of the bacillus bulgaricus. J. Infect. Dis., **6**, 304.

HEINEMANN, P. G. AND ECKER, E. E. 1916 A study of the Boas-Oppler bacillus. J. Bact., **1**, 435.

KENDALL, A. I. 1910 Observations on aciduric (acidophilic) bacteria. J. Med. Research, **22**, 153.

LLOYD, D. 1917 On the chemical factors involved in the growth of the meningococcus. Brit. Med. J., **1**, 11.

NOGUCHI, H. 1910 Pleomorphism and pleobiosis of bacillus bifidus communis. J. Infect. Dis., **12**, 182.

RAHE, A. H. 1914 An investigation into the fermentative activities of the aciduric bacteria. J. Infect. Dis., **15**, 141.

RAHE, A. H. 1915 A study of the so-called implantation of the bacillus bulgaricus. J. Infect. Dis. **16**, 210.

ROGERS, L. A. AND DAVIS, B. J. 1912 Methods of classifying the lactic acid bacteria. U. S. Dept. Agriculture, Bureau of Animal Industry, Bulletin, 154.

TORREY, J. C. AND RAHE, A. H. 1915 A new member of the aciduric group of bacilli. J. Infect. Dis., **17**, 437.

TORREY, J. C. 1917 New differential plating methods for B. bifidus (Tissier) and B. acidophilus (Moro). J. Bact. **2**, 435.

WHITE, B. AND AVERY, O. J. 1910 Observations on certain lactic acid bacteria of the so-called Bulgarian type. Centralbl. f. Bakt., b. **25**, II. Abt., 161.

THE GERMICIDAL ACTION OF FREEZING TEMPERATURES UPON BACTERIA

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Temperature is one of the cardinal factors influencing life activities of micro-organisms. The majority of bacteria are unable to exercise normal metabolism at temperatures below 6°C. or above 45°C.

Temperatures above the maximum are injurious to bacteria, and any appreciable increase above this critical point leads to death. Death of bacteria, due to high temperature, is not instantaneous, but proceeds in an orderly fashion at a rate which is predictable, though very rapid increases in the degree of heat may accelerate death so that it appears immediate. This has been explained as the outcome of accelerated metabolism which leads to auto-destruction. The destructive influence of other disinfectants, both physical and chemical, proceeds in a similar manner and may have a similar explanation.

Cold as a disinfectant seems to be an exception to this rule; in fact it has the opposite effect, as metabolism is arrested to its lowest ebb by temperatures lower than the minimum, and just below this critical point survival should, theoretically, be at its maximum. When the temperature is depressed to the freezing point or below, new factors enter into the problem and it is with these that this paper is primarily concerned.

The influence of low temperatures on micro-organisms has received relatively little attention. In 1882 Pumpelly found that samples of ice cut from the center of the block and inoculated into sterile beef broth showed living contamination.

The first extensive investigations were made by Prudden in 1887. Pure cultures of *B. prodigiosus* and *B. proteus* were found to be sterile after fifty-one days at temperatures ranging between $-10°$ to $1°C$. *B. typhosus* survived for at least one hundred and three days at a temperature between 14° and 30°F. It will be of interest in relation to the data presented later to reproduce the results obtained by alternate freezing and thawing.

By coating tubes with sweet oil to prevent the crystallization of the water, and comparing the death rates in these tubes with

TABLE 1

*Continuous freezing compared with alternate freezing and thawing**

FROZEN SOLID		ALTERNATE FREEZING	
B. typhosus			
Before freezing	40,896	Before freezing	40,896
Frozen 24 hours	29,780	Frozen 3 times	90
Frozen 3 days	1,800	Frozen 5 times	0
Frozen 4 days	950	Frozen 6 times	0
Frozen 5 days	2,490		
B. prodigiosus			
Before freezing	339,516	Before freezing	339,516
Frozen 24 hours	36,410	Refrozen 1 time	2,570
Frozen 30 hours	41,580	Refrozen 2 times	275
Frozen 48 hours	14,440	Refrozen 3 times	15
Frozen 96 hours	4,850	Refrozen 4 times	0

* Frozen at 2° to 5° F., then kept just below 32° F.

those in which solid freezing had occurred, the former showed greater fatality. Prudden concludes that "the greatest reduction occurs during, or shortly after the sudden reduction of temperature to freezing, and, if after this the bacteria remain in ice, a comparatively gradual destruction goes on; if bacteria are thawed out and immediately refrozen another large increment is destroyed." The killing action of cold as such is chiefly emphasized.

Ravenel, (1899) Macfayden, and others have obtained very low temperatures with liquid air and liquid hydrogen (−252)

and have concluded in general that cold cannot be depended upon to sterilize.

Park (1901) records 100 per cent reduction with twenty different strains of typhoid subjected to −5°C. for twenty-two weeks. From their extensive work Sedgwick and Winslow (1902) concluded that not only different species but different "races" within the same species exhibit marked variability in their resistance to freezing temperatures.

Keith, (1913) contrary to Prudden, has emphasized the importance of solid freezing as compared with cold in relation to the death rate of bacteria. *B. coli* frozen solidly in water at −20°C. show 99 per cent killed in five days, but when not actually frozen a large per cent remain alive for months; when frozen in diluted milk the death rate increases with the dilution; when suspended in aqueous mixtures of 5 to 42 per cent glycerine a large percentage remain alive for six months at −20°C. Keith concluded that the important factors influencing the death rate of bacteria at low temperatures are their rate of metabolism and the mechanical protection offered by the medium.

In 1915 one of the authors in collaboration with Torossian and Stone published notes on the germicidal effect of freezing and low temperatures. We suggested there that "bacteria may be killed by the mere fact of low temperature, interfering with metabolism; by freezing of the cell contents and rupture of the membrane by internal pressure; by external pressure or grinding developed during crystallization; or by expansion of the frozen medium within the receptacle; or by more or less prolonged suspension of metabolic activities, leading to slow death from old age or starvation." Below we reproduce the tables as given in these notes. The extension of the work since this time has entirely confirmed this preliminary study as regards (a) the rapid destruction of 90 per cent or more of *B. coli* when frozen in tap water for three hours, (b) the greater viability of spores (*B. subtilis*) in frozen mixtures, (c) the greater germicidal influence of intermittent freezing and thawing, (d) the fact that depression of the temperature within certain limits

increases slightly the germicidal activity, (e) and the fact that cream and milk furnish some protection to bacteria frozen in them either continuously or intermittently.

We were most interested to learn, if possible, whether some external factor, other than the low temperature, entered into the

TABLE 2

A comparison of the percentage reduction of B. coli held at 0.5°C., −15°C., and frozen intermittently in tap water for a three hour period

INITIAL COUNT	FIRST FREEZING	SECOND FREEZING	THIRD FREEZING	FOURTH FREEZING	CONTINUOUS FREEZING THREE HOURS −15°	COLD 0.5°C THREE HOURS
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2,130	82.2	99.9	99.9	99.9	99.9	23.0
1,650	92.8	96.1	99.8	99.9	99.7	29.0
1,320	93.8	98.7	99.9	99.9	99.4	47.0
3,015	97.6	99.6	99.5	99.9	99.8	31.3
4,800	98.6	99.4	99.8	99.8	99.8	32.0
1,370	98.6	99.5	99.8	99.9	99.7	8.1
1,070	97.9	99.5	99.5	99.9	99.9	97.3

TABLE 3

Percentage reduction obtained with B. coli in cream at freezing temperatures

INITIAL COUNT	FIRST FREEZING	SECOND FREEZING	THIRD FREEZING	FOURTH FREEZING	THREE HOURS FREEZING 0°C.	COLD 0.5°C. THREE HOURS
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
4,350	4.8	39.3	45.0	48.9	61.3	18.0
4,740	40.5	45.5	71.5	75.9	67.7	42.7
5,275	43.1	46.7	71.9	81.2	44.2	16.4
5,284	33.4	48.2	60.2		26.4	20.8
5,028	32.2	36.2	48.4	71.7	34.8	20.6
3,732	35.2	20.9	42.3	50.1	33.6	38.9
4,030	71.0	67.1	78.6	83.1	67.6	3.9
5,085	21.1	51.6	53.3	75.4	65.3	23.8
4,725	16.1	36.5	52.2	72.6	58.0	16.8
4,560	34.8	47.1	67.4	63.8	54.2	19.7

destruction of the bacteria. Is there a critical degree of cold at, or, just below, freezing which is highly fatal, or is the crystallizing action itself destructive? We have noted the conclusion of Prudden, where cold itself was chiefly emphasized. Keith emphasized the solidification. Most work on cold has been

tested over a long period of time without relation to the quantitative aspect, while we have studied chiefly its influence over very short periods and have consistently made quantitative studies.

In order to eliminate crystallization and possible mechanical crushing of bacteria during the freezing of the medium, the freezing point was depressed by the addition of a non-electrolytic substance, grape sugar. From the formula given in Harper's Scientific Memoirs for the lowering of the freezing point of water in degrees Centigrade, produced by dissolving a gram molecule of a given substance in a liter of water, it was possible to

TABLE 4

	GLUCOSE
	grams
To depress the freezing point to −6°C	56.2
To depress the freezing point to −4°C	37.5
To depress the freezing point to −3°C	28.1
To depress the freezing point to −2°C	18.7
To depress the freezing point to −1.5°C	14.0
To depress the freezing point to −0.5°C	4.7

calculate the amount of glucose ($C_6H_{12}O_6$) which must be added to a liter of water to depress its freezing point by any desired increment. The table above gives the amount of glucose required to depress the freezing point to a certain degree as worked out by the above formula.

The several sugar solutions were made with chemically pure glucose and sterilized in streaming steam on three successive days. The pure cultures of *B. coli*—six strains in all were used —were frequently transferred and a twenty-four hour old culture in standard bouillon was always used in the tests. In all of the experiments sterile tap water was used as a control, and for comparison. The culture was inoculated into the tubes of water and solutions to be tested, the initial numbers determined by plating from each tube in several dilutions, always using duplicate plates, and then the tubes were immersed in an ice-

salt bath of a concentration to give approximately the temperature desired, and the temperature was watched for the period of the experiment and adjusted when necessary. Melting of frozen tubes was brought about gradually by immersion in cold water. All plates were incubated at 37°C. and counted after forty-two hours.

To determine that the glucose in the concentrations used was not germicidal, either on account of its chemical or osmotic properties, each solution was inoculated and placed in the refrigerator at from 4 to 6°C. for three hours. At least four tests for

TABLE 5

CONCENTRATION OF SUGAR	TEMPERATURE	SUGAR			WATER		
		Initial count	Final count	Reduction	Initial count	Final count	Reduction
grams per liter	*deg. C.*			*per cent*			*per cent*
4.7	−0.5	880	515	41.4	790	150	81.0
		613	378	38.3	682	109	85.4
		132	55	58.3	55	0	100.0
		650	300	53.8	435	6	98.6
		414	226	45.4	575	10	98.2
		150	88	41.3	1,340	18	98.6
			Average	49.6		Average	90.3
14.0	−1.5		Average	36.2		Average	95.9
18.7	−2.0		Average	40.8		Average	93.1
28.1	−3.0		Average	44.2		Average	96.9
37.5	−4.0		Average	49.3		Average	98.8
56.2	−6.0		Average	49.5		Average	99.2

each solution were made and the control in tap water was made each time. The reductions in sugar solutions were variable but were uniformly lower than those which occurred in water, so we feel entirely justified in concluding that the sugar as such had no germicidal influence.

We then undertook to compare the effect of low temperatures upon bacteria in a medium which crystallized, with one where crystallization was absent. The detailed data are given for the series of experiments at one temperature only and the average reduction of a similar series of experiments at each of the other temperatures is given in table 5.

Comparing the percentage reduction which occurs in the fluid sugar solutions, and the solidified water kept at the same temperature for the same period of time, and with all other factors as nearly identical as possible, it is readily seen that the death-rate is much higher in the solidified tubes. This indicates a very conspicuous rôle played by crystallization as such, regardless of the factor of cold.

This was tested still farther by actually freezing the strongest sugar solution to make certain that the solute itself did not somehow fortify against cold. Some of the results are given in table 6.

In this table the reductions obtained in the solidly frozen sugar solutions are uniformly higher than those obtained in the

TABLE 6

B. coli frozen solid at −10°C. for three hours in a solution of glucose 56 grams per liter and in tap water

Sugar			Water		
Initial count	Final count	Reduction	Initial count	Final count	Reduction
		per cent			*per cent*
633	50	92.1	55	1	98.1
855	8	99.0	318	1	99.6
2,900	32	98.8	1,340	2	99.8
7,560	1,700	77.5	5,770	87	98.4

previous results and are quite comparable with the reductions in the water controls.

From the foregoing discussions and data we venture to draw certain conclusions, appreciating, however, that the work is not extensive enough to render any of these statements final.

1. Intermittent freezing of bacteria exerts a more effective germicidal action than continuous freezing.

2. The reduction is much less in milk and cream than in pure tap water when freezing temperatures are applied, due, no doubt, to physical protection offered to the bacteria by the colloidal and solid matter in suspension.

3. The degree of cold below freezing is not a very important factor in the destruction of bacteria. There is no critical

temperature below freezing where the germicidal effect is greatly accelerated.

4. The death-rate of *B. coli* is much higher in media which are frozen solid than it is in the same media not solid and at a slightly lower temperature.

5. Crystallization, probably resulting in mechanical crushing, is an important germicidal factor in causing the death of bacteria at zero degrees Centigrade and below. The greatest reduction occurs promptly upon freezing and refreezing, but is not caused so much by the sudden change in temperature as by this mechanical factor.

REFERENCES

Albert, Hinman and Jordan 1916 Bacterial changes in an uniced specimen of water. J. Bact. **1**, 119.

Back, E. A., and Pemberton, C. E. 1915–16 Effect of cold storage temperatures upon the Mediterranean fruit fly. J. Agric. Research, **5**, 657.

Bartram, H. E. 1915–16 Effect of natural low temperatures on certain fungi and bacteria. J. Agric. Research, **5**, 651.

Conn, H. J. 1914 Bacteria of frozen soil. N. Y. Agric. Exp. Sta. Tech. Bull. 35, July, 1914.

Conn, H. W., and Esten 1904 Changes undergone by milk at low temperatures. Report, Storrs Agric. Exp. Sta. 1904, p. 27.

Hale, F. E., and Melia, T. W. 1910 Studies on inhibition, attenuation, and rejuvenation of B. coli. J. Infect. Dis. **7**, 587.

Hilliard, C. M., Torossian, C., and Stone, R. 1915 Notes on the factors involved in the germicidal effect of freezing and low temperatures. Science, **42**, Nov. 26, 1915.

Keith, S. C. 1913 Factors influencing the survival of bacteria at temperatures in the vicinity of the freezing point of water. Science, **41**, June 6, 1913.

Kellicott 1916 Effects of low temperature on the development of fundulus. Am. J. Anat., **20**, 449.

Kendall, A. I. Bacteriology, Chap. II, p. 42.

Kolle and Wasserman 1911 Handbuch der Pathogenen Mikroörganismen. Vol. II, p. 30 Milzbrand Physikalische Schädigungen.

Park, W. H. 1901 Duration of life of typhoid bacilli in ice. Effect of intense cold on bacteria. Science, Mar. 1, 1901, No. 322, p. 323.

Pennington, M. E. 1908 Bacterial growth and chemical changes in milk held at low temperatures. J. Biol. Chem. 1908, p. 353.

Pengra, C. P. 1884 Purification of water by freezing. 12th Report Michigan State Board of Health

Pictet and Young 1884 De l'action du froid sur les microbes. Compt. rend. Acad. d. Sc., **98**, 747.

PRUDDEN, T. E. 1887 On bacteria in ice and their relation to disease. Med. Rec., March 26 and April 2.

PUMPELLY, RAPHAEL 1882 The dangers of impure ice. Sanitarian, May, 1882.

RANSOM, B. H. 1914–15 The destruction of the vitality of Cysticercus bovis by Freezing. J. Parasitol. **1**, 5–9.

RANSOM, B. H. 1916 The effects of refrigeration upon the larvae of Trichinella spiralis. J. Agric. Research, **5**, 819.

RAVENEL, M. P. 1899 The resistance of bacteria to cold. N. Y. Med. News, **74**, June 10.

RAVENEL, M. P., HASTINGS, E. G., AND HAMMAR, B. W. 1910 The bacterial flora of milk held at low temperatures. J. Infect. Dis. **7**, 38.

Report of Commission on Cold Storage, Bell. 1916. Am. J. Pub. Health, **6**, 1119.

Scientific Memoirs. The theory of solutions. Harry C. Jones, Ed.

SEDGWICK, HAMILTON AND FUNK 1917 Experimental studies on the effect of various solutions upon the viability of bacteria at low temperatures. Abst. Bact. **1**, 49.

SEDGWICK, W. T., AND WINSLOW, C.-E. A. 1902 Experiments on the effect of freezing and other low temperatures on vitality of bacillus of typhoid fever. Mem. Acad. Arts and Sc. **12**, (5).

WILEY, H. W. 1908 Effects of cold storage on eggs, quail, and chickens. Bull. 115, Bur. of Chem.

A SYNTHETIC MEDIUM FOR THE DIRECT ENUMERATION OF ORGANISMS OF THE COLON-AEROGENES GROUP

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The direct numerical determination of the organisms of the colon-aerogenes group has attracted the attention of numerous investigators and several different media have been originated for this purpose. There are many difficulties which are encountered in an attempt to devise a selective medium upon which organisms of this group will grow while other species of bacteria are excluded. The difficulties are too well recognized by all who have attempted direct quantitative determinations to warrant further discussion.

The media commonly used for the direct determination of the colon-aerogenes group are very complex and depend largely for their success upon some constituent which inhibits the non-gas forming lactose fermenting organisms. This seems to be the wrong principle. Instead of depending upon inhibition of certain species it is believed that it is far better to simplify the medium to a point where the sources of nitrogen and carbon are in such a form as to allow only the growth of the organisms desired. With this view in mind a simple synthetic medium has been devised in which there is a single source of nitrogen, namely, sodium ammonium phosphate and a single source of carbon, namely, lactose.

COMPOSITION OF THE MEDIUM

Solution I

	per cent
Sodium ammonium phosphate	0.4
Acid potassium phosphate	0.2
Lactose	1.0

Dissolve in distilled water.

Solution II

Filtered solution of agar in distilled water	3.0

Mix solution I and II in equal proportions while hot and put up in definite amounts of 100 cc., or more, in flasks or bottles and then sterilize. The 3 per cent agar solution is made up separately and kept in stock merely for convenience. Agar can be added directly to solution I at the time of preparation if desired, using 1.5 per cent. A slight precipitate may appear upon sterilization, but this does not interfere with the count and may not appear on the plate.

INDICATOR

To indicate the acid colonies basic fuchsin is employed and decolorized by sodium sulfite using the following solutions:

Solution A. One per cent alcoholic solution of basic fuchsin.

Solution B. Five per cent aqueous solution sodium sulfite, freshly prepared.

At the time of plating, after the agar medium is melted and while at its highest temperature add for each 100 cc. of medium 0.5 cc. of a 1 per cent alcoholic basic fuchsin solution and follow at once with 0.5 cc. of a freshly prepared 5 per cent sodium sulfite solution. Mix thoroughly and allow to cool, then pour into plates as usual. The red color is not entirely destroyed by the sulfite and at time of plating the medium is pink.

Purified litmus or brom cresol purple can be used as indicators, but if more than about thirty colonies appear on the plate the entire medium changes to the acid color of the indicator and it then becomes impossible to detect acid colonies.

With decolorized basic fuchsin there is a red coloration or at least a deep red colony which is probably caused by the combined action of acid and aldehyde as has been shown by De Bord (1917). This indicator is preferred, therefore, to the two previously mentioned, because the acid-forming organisms of the colon-aerogenes group produce deep red colonies which can be detected when present in any countable number even when the medium has become entirely red.

INCUBATION

Plates may be incubated at 30°C. or at 37°C. and should be counted after forty-eight hours. The colonies appear somewhat more quickly and are larger at the higher temperatures. If the plates are incubated longer than forty-eight hours some bacteria which do not belong to the colon-aerogenes group may develop. Upon incubation the medium becomes red, but there is no difficulty in distinguishing the characteristic deep red colonies.

APPEARANCE OF COLONIES OF ORGANISMS OF THE COLON-AEROGENES GROUP

The colonies appear on the plates as medium sized red colonies with a deep red ring around them. The color about the colony is quite characteristic, although some have only a deep red color in the colony itself.

Bacteria which may also grow upon the medium and which do not belong to the colon-aerogenes group usually give pink or uncolored colonies which can be readily distinguished from those of the gas formers. Before this medium is used for routine work it is recommended that pure cultures of the organisms of the colon-aerogenes group be plated on the medium in order that the appearance of their colonies may be observed.

SELECTIVE ACTION OF THE MEDIUM

The synthetic medium has a selective instead of inhibitory action for two reasons. First, it allows only the development of bacteria which can obtain their nitrogen from a very simple compound, such as sodium ammonium phosphate. This naturally prevents the growth of a large number of species of bacteria. Second, the only source of carbon is lactose which therefore allows only the growth of bacteria which ferment this sugar.

A large number of organisms of the colon-aerogenes group have been tried and all found to be capable of utilizing nitrogen and carbon from the above mentioned sources. Relatively few other species of bacteria were encountered which could grow on

this medium and only occasionally were colonies of non-gas forming organisms observed which resembled those of organisms of the colon-aerogenes group.

Certain molds may develop on this medium which first appear as minute red colonies, but later they show the typical appearance of mold colonies.

It is not claimed that the medium is perfect in its selective action, that is to say, that it allows only the growth of organisms of the colon-aerogenes group. Probably a medium perfect in this respect can never be obtained. Every colony which develops can not be considered a gas former, but from the appearance of the colonies it is believed an accurate direct colon count can be obtained.

The flora of the material under examination may influence the accuracy of the count and the value of this simple synthetic medium. It has proven of great value in the determination of the colon count of milk and undoubtedly it can be used in the examination of other foods. A few results indicate that the medium should be particularly useful in the direct enumeration of organisms of the colon-aerogenes group in water.

This synthetic medium can probably be improved and it is hoped that it will be given a thorough trial in different laboratories and its defects brought to the attention of the writers.

ADVANTAGES OF THE MEDIUM

1. Apparent accuracy in the direct enumeration of bacteria of the colon-aerogenes group.
2. Constancy of composition.
3. Simplicity of preparation.
4. Cheapness.

REFERENCE

De Bord, George C. 1917 The fuchsin aldehyde reaction on endo medium. Jour. Bact, 2, 309–314.

THE ENDO MEDIUM FOR THE ISOLATION OF B. DYSENTERIAE AND A DOUBLE SUGAR MEDIUM FOR THE DIFFERENTIATION OF B DYSENTERIAE, SHIGA AND FLEXNER[1]

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This paper is based on a study of means of isolating *Bacillus dysenteriae* from human excreta and polluted soil or water, in which it may be contained. Previous experience (Kligler, 1918) had shown that with respect to sensitiveness to chemicals, the dysenteric group of bacilli approached the Gram positive bacteria; or, in other words, departed from the typhoid and paratyphoid groups. On the other hand, when an enriching medium was employed for cultivation (nutrose, bile, egg), it proved just as favorable to the growth of other intestinal bacteria as to *B. dysenteriae.* Hence resort was had to a plate medium that would afford colony differentiation and permit at the same time the development of very delicate strains of the dysentery bacillus. Of the two available media for this purpose, litmus-lactose agar and Endo's medium, the latter presented certain advantages.

Modified Endo medium. In carrying forward the cultivation on the Endo medium it was discovered that pure cultures of the Shiga dysentery bacillus or cultures in fecal emulsions sometimes gave irregular results. It appeared at first that the fuchsin acted as the inhibitive agent, being more effective against the Shiga bacillus than against the Flexner group of bacilli. In following out this line of experiment, it was determined that the

[1] Work aided by a grant from the International Health Board of the Rockefeller Foundation.

addition either of nutrose or bile produced a marked improvement of the medium, the former surpassing the latter in this respect. Further study showed, however, that other factors were concerned, and finally, by elimination and new cultivation, a medium was evolved which offered distinct advantages for the cultivation of the dysentery bacilli. The modification consists in the substitution of sodium bisulphite as recommended by Robinson and Rettger (1916) and the accurate adjustment of the hydrogen ion concentration to a P_H value of 7.6–7.8. The medium as ordinarily prepared with a phenolphthalein reaction of +0.2 gives an end reaction, after the addition of the sodium-sulphite which is alkaline, ranging from P_H 8.4 to 8.8. This degree of alkalinity is inhibitive to the growth of the Shiga bacillus.

Either meat-infusion or beef-extract agar may be used. We use a beef-extract medium prepared as follows:

Peptone	10.00 grams
Beef extract	3.00 grams
NaCl	5.00 grams
Agar	15.00 grams
Water	1000 cc.

All the ingredients except the agar are dissolved first; the agar is then added and the mixture autoclaved for one hour at 15 pounds pressure. It is then cooled to 50°C., white of egg added (2 eggs to 5 liters), and steamed in the Arnold for thirty minutes. The reaction is then adjusted to P_H 7.4, with phenolsulphonephthalein as the indicator, the medium boiled on the free flame for six to seven minutes, filtered, flasked, and autoclaved.

This constitutes the stock[2] medium from which all the special media—brilliant green, Endo, etc.—may be prepared. Before pouring the Endo plates the reaction is adjusted to P_H 7.6–7.8, and the lactose and the fuchsin-sulphite are added in the usual manner. It has been our experience that about 0.8 cc. N NaOH

[2] This agar can be used for all purposes. The reaction is favorable for the growth of all common bacteria. In places where a large amount of medium is used, and in the field, it is especially advantageous to have a single stock that can serve as a basis for the various modified media.

was required for every 100 cc. of agar. It is necessary, however, to determine the exact amount for each fresh lot of agar.

Double sugar medium. Having developed the colonies on the Endo plate, it is necessary to carry the differentiation further so as to confirm the nature of the selected colonies. Russell (1911) makes use of a double sugar agar containing 0.1 per cent glucose and 1 per cent lactose. While this medium is effective, it does not permit the distinction of *B. typhosus* and the two classes—alkaline or Shiga and acid or Flexner, etc.—of dysentery bacilli from each other. We have substituted 0.5 per cent mannite for the lactose, with the effect of accomplishing the purpose.

The selected colonies are stabbed in the butt of the slanted tube and also streaked on the surface. Andrade's indicator is used. The differentiation is made after twenty-four hours' incubation at 37°C. The Shiga bacillus yields a slight reddening of the butt but no change of the surface. The acid class of dysentery bacilli and *B. typhosus* color the entire medium red; no gas is produced. *B. alcaligenes* produces no changes; while the paratyphoid bacilli cause both reddening and gas formation. *B. proteus*, on the other hand, produces gas but no reddening. If decolorized fuchsin is employed as indicator, then the paratyphoid bacilli A and B can be distinguished from each other, as the latter brings about final decolorization of the medium. However, the main advantage of this medium is that it permits of a more rapid separation of the two classes of dysenteric bacilli—the Shiga and the Flexner.

SUMMARY

A study of the Endo medium, as applied to *B. dysenteriae*, indicated that the most important single condition that must be carefully controlled, particularly if the medium is intended for the isolation of dysenteric bacilli, is the end reaction. The ingredients are of singificance in so far as they furnish the elements essential for the growth of the organisms. Given an otherwise favorable Endo medium, however, the Shiga bacillus will or will not grow, depending on whether the end reaction is P_H 7.6–7.8 or P_H 8.4–8.8, the reaction ordinarily obtained.

A mannite-glucose double sugar medium on the principle of Russell's double-sugar agar is described, to be employed for rapid differentiation of the Shiga and Flexner class of dysenteric bacilli.

REFERENCES

Kligler, I. J. 1918 J. Exp. Med., **27**, 463.
Russell, F. F. 1911 J. Med. Res., **25**, 217.
Robinson, H. C., and Rettger, L. F. 1916 J. Med. Res., **34**, 363.

NOTE ON CROSS-AGGLUTINATION OF B. COLI COMMUNIS AND B. DYSENTERIAE SHIGA[1]

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This note refers to an instance of cross-agglutination between two distinct bacilli in immune sera, prepared for each, and in practically identical end dilutions.

Other similar instances of cross-agglutinations are reported in the literature, but with few exceptions either the agglutination limit was less for the heterologous than for the homologous organism, or the coöperation of the two organisms in producing the agglutinins could not be excluded. Thus Stern (1898) studied five samples of blood serum from patients with typhoid fever which agglutinated, equally with the typhoid bacilli, the cultures of *B. coli* isolated from their stools. In this example, the possibility exists that mixed infection with the colon bacilli coexisted. Rodet (1897) found that sheep sera derived from animals immunized with *B. typhosus* and *B. coli*, respectively, agglutinated the heterologous organism equally with the homologous. Park and Williams (1910) observed the serum of a horse immunized with the Flexner dysenteric bacilli which agglutinated a culture of *B. coli* in the same end dilutions (1: 10,000) as the dysenteric bacillus. Conversely, a goat immunized with the *B. coli* culture yielded a serum of a titre of 1: 5,000 for the *B. coli* and 1: 3,000 for Flexner *B. dysenteriae*. The chief interest of the following communication arises from the fact that it concerns a similar cross-agglutination of *B. coli* and *B. dysenteriae* Shiga.

[1] Work conducted under a grant of the International Health Board of the Rockefeller Foundation.

EXPERIMENTAL

The culture of the *B. coli* used in the tests was recently obtained from the stool of a chronic carrier of *B. typhosus*. On inoculation into rabbits it proved pathogenic, but never induced the paralysis characteristic of the Shiga bacillus inoculation of that animal. The Shiga bacillus had been cultivated outside the body for a

TABLE 1

Particular and general agglutination tests with special B. coli and B. dysenteriæ sera

CULTURE		DYSENTERY SERUM						COLON SERUM					
		100	500	1000	2000	4000	C	100	500	1000	2000	4000	C
B. dysenteriæ (Shiga)	30	++	++	++	+	±	−	++	++	++	++	+	−
	6	++	−	−	−	−	−	+	−	−	−	−	−
	27	−	−	−	−	−	−	−	−	−	−	−	−
B. coli	13	++	++	++	+	±	±	++	++	++	++	+	±
	11	−	−	−	−	−	−	±	−	−	−	−	−
	12	−	−	−	−	−	−	−	−	−	−	−	−
	14	−	−	−	−	−	−	−	−	−	−	−	−
	95	−	−	−	−	−	−	−	−	−	−	−	−
	104	−	−	−	−	−	−	−	−	−	−	−	−
	137	−	−	−	−	−	−	−	−	−	−	−	−
B. dysenteriæ	2	−	−	−	−	−	−	±	−	−	−	−	−
(Flexner)	22	−	−	−	−	−	−	−	−	−	−	−	−
	23	−	−	−	−	−	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−	−	−	−	−	−
	25	−	−	−	−	−	−	−	−	−	−	−	−
	26	±	−	−	−	−	−	−	−	−	−	−	−
	28	−	−	−	−	−	−	−	−	−	−	−	−
	29	−	−	−	−	−	−	−	−	−	−	−	−
	S	−	−	−	−	−	−	−	−	−	−	−	−

long time; the original culture was obtained from Dr. Wadsworth of the New York State Department of Health. It fulfilled all the morphological and cultural requirements and upon inoculation into rabbits induced the typical paralysis. The two organisms had the property in common of tending to spontaneous agglutination.

The property of reciprocal agglutination was limited to the two strains described. Other cultures of *B. coli* and *B. dysenteriae* Shiga did not exhibit it. That is, agglutinating sera prepared with the particular strains of the colon and Shiga dysentery bacilli mentioned reacted on each other quite as on their own cultures, while other colon bacilli showed no especial agglutinating capacity with the Shiga immune serum nor did other Shiga bacilli, with the colon agglutinating serum. Similarly the stock poly-

TABLE 2

Absorption tests for agglutinins performed with homologous and heterologous cultures

SERUM	ABSORBING CULTURE	AGGLUTINATION TEST CULTURE	DILUTIONS 250	500	1000	2000	4000	8000	C
30	13	13	–	–	–	–	–	–	–
		30	++	++	+	±	±	–	–
	30	13	±	–	–	–	–	–	–
		30	+	–	–	–	–	–	–
13	13	13	±	–	–	–	–	–	–
		30	+	–	–	–	–	–	–
	30	13	++	++	++	++	++	–	–
		30	++	+	–	–	–	–	–
Controls.... 30	None	13	++	++	++	+	–	–	–
		30	++	++	++	++	–	–	–
Controls.... 13	None	13	++	++	++	++	++	–	–
		30	++	++	++	++	+	±	–

valent dysenteric horse serum agglutinated the particular *B. coli* strain at 1: 2000, which was its value to its homologous serum.

Single colonies of each organism were selected after several platings and employed to immunize rabbits. The injections were given at three day intervals. The rabbit receiving the culture of *B. coli* was given five intravenous injections, beginning with 1/100 and ending with 1/10 of an agar slant of living culture. The rabbit receiving the culture of the Shiga bacillus received seven intravenous injections, beginning with 1/50th

of a slant of a killed culture and ending with 1/10th of the living culture.

The results with the sera prepared from isolated colonies of each bacillus coincided with those of the original test. The fact should be mentioned that the particular Shiga immune serum was without agglutinating action on other Shiga cultures or cultures of the Flexner dysenteric group. Table I brings out in brief form the points just described. The cross-agglutinating colon bacillus culture is number 13 and the Shiga bacillus culture number 30.

Absorption of the immune sera was carried out with the homologous and heterologous agglutinating cultures, after which agglutination tests were made. The results are given briefly in table 2.

SUMMARY

Two cultures are described, one a typical *B. coli* and the other a typical *B. dysenteriae*, Shiga, culture, which yield immune sera possessing agglutinating properties of practical equal quantity for each culture. Absorption experiments made with each culture upon each kind of immune serum indicate that two distinct agglutinins are yielded in about equal amount in the process of immunization of rabbits with the respective cultures. The two agglutinins are specific ones, each for its own culture, and accessory (paragglutinin), each for the other culture. The absorption of the accessory agglutinin leaves the specific agglutinin quantitatively unaffected.

The significance of this reciprocal agglutinative property in respect to the two bacilli described can only be surmised. The obvious suggestion is a group relationship between certain strains of colon and dysenteric bacilli, a subject hardly to be pursued profitably in this connection.

REFERENCES

Castellani, A. 1902 Ztschr. f. Hyg., **40**, 1.
Park, W. H., and Williams, W. 1910 Pathogenic Microorganisms, 166, 168.
Rodet, A. 1897 Compt. rend. de la Soc. de Biol., **4**, 874.
Stern, R. 1898 Centralb. f. Bakt., **23**, 673.

COMMENTS ON THE EVOLUTION AND CLASSIFICATION OF BACTERIA

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The incompleteness of our present knowledge as to the species of bacteria and their relationships makes it very easy to criticise what others have written upon their evolution and to propose modifications in the classification of the group which in their turn are open to equal criticism. There has been so much speculation along this line recently[1] that a certain amount of confusion has resulted, and there is danger of increasing this confusion by discussing the subject from a new point of view. There would be little justification, indeed, for doing so, were it not for the fact that Jensen (1909) has already drawn up a detailed ancestral tree for the known groups of bacteria which has considerable merit and that the committee on classification appointed by the Society of American Bacteriologists (Winslow, et al., 1917b) has accepted many of Jensen's suggestions. This committee has given the matter so much thought and has drawn up such a well formulated report that there is danger of their recommendations being accepted *in toto* without sufficient scrutiny. Underlying their classification are certain assumptions as to the evolution and relationships of bacteria that should be thoroughly discussed before the report is adopted by the society.

In discussing the relationships of bacteria, it is necessary to keep in mind—just as when dealing with higher forms of life—that the living species represent only the ends of evolutionary lines, and that one modern form must not be considered the ancestor of another. It is probably true that there has been a greater persistence of primitive types among bacteria than in any other group of animals or plants, because the environment

[1] See Jensen (1909), Buchanan (1917) and Kligler (1917).

of many bacteria—salt water, fresh water and soil—has presented fairly uniform conditions throughout long geologic periods. Yet we can hardly assume that all of the primitive types of bacteria are still living. In fact, it would be more in harmony with what is known of the persistence of species and genera among higher forms of life—especially when the very brief life-cycle of bacteria is considered—to assume that the primordial types are all extinct. This idea seems to have been overlooked by Kligler in his recent paper (1917). Jensen in general kept the fact well in mind that he was dealing with end-products only; yet he also overlooked it at times, and failed to incorporate it into the diagram which he drew up to show the relationships of the different groups. In this diagram Jensen places the cephalotrichic organisms at the base of three main lines of development instead of representing them as a main branch coördinate with the peritrichic forms. Similarly Kligler makes no attempt to indicate that the Pseudomonas line of development appears to be as diverse as any other line; and has even indicated modern pathogenic forms (diphtheroids and albococci) as the ancestors of micrococci, a group which presumably includes the primitive cocci of soil.

This fallacious line of reasoning has led to certain questionable conclusions as to the relationships of bacteria, some of which have even been accepted by the committee on classification. The best way to point out this weakness in their report is to examine critically the different groups which they propose to establish.

The orders. The committee on classification recognizes four orders, Myxobacteriales, Thiobacteriales, Chlamydobacteriales, and Eubacteriales, together with an appendix, Spirochaetaceae. It is to be noted that they place Eubacteriales last, although it contains the simplest forms, while it is customary to arrange classifications in such a way that the simpler groups are placed at the beginning. The committee, indeed, has used the latter plan in arranging the classification of the Eubacteriales, as their first family, Nitrobacteriaceae, is the one they consider most primitive. In this respect there is an evident inconsistency

between the arrangement of their orders and the arrangement of their families.

Buchanan, in his recent classification (1917), recognizes six orders instead of four. He obtains this number by giving ordinal rank to the Spirochaetes and Actinomycetes. There is considerable reason to question whether the Spirochaetes belong with the Schizomycetes or with the Protozoa, so perhaps the committee's treatment of them is as satisfactory as Buchanan's. The Actinomycetes, however, differ so decidedly from true bacteria and embrace such a diversity of species[2] that there is good reason for accepting Buchanan's suggestion that there be a separate order Actinomycetales.

In passing, it is of interest to notice that there is a close analogy between the generally recognized groups of bacteria and those of Protozoa. Thus the cephalotrichic and peritrichic bacteria find their analogues respectively in the Flagellates and Ciliates (Infusoria). This analogy goes further than a mere resemblance in the arrangement of organs of locomotion; for the Ciliates and the peritrichic bacteria are both highly specialized groups, while both Flagellates and cephalotrichic bacteria contain all gradations between primitive forms and highly specialized human parasites. There is also a less striking analogy between the Rhizopods and the cocci, both groups with equal diameters. More striking is the resemblance between the two highly specialized, but apparently unrelated groups, the Myxobacteria and the Mycetozoa. Probably this analogy has no greater significance than the similar one so often mentioned between marsupials and placental mammals, in both of which groups the lines of development show much superficial similarity. Under similar conditions, different groups of living things frequently appear to undergo evolutionary development along parallel lines. This similarity between the groups of bacteria and of Protozoa does not indicate interrelationship; but it does increase the probability that the orders of bacteria just men-

[2] The diversity of species of Actinomycetes has been discussed by Lachner-Sandoval (1898), Neukirch (1903) and Conn (1917).

tioned represent the lines of development of the bacteria more truly than the physiological groups proposed by Jensen.

It is next necessary to take up in turn the seven families into which the committee has divided the order Eubacteriales.

Family 1. Nitrobacteriaceae. The justification for the recognition of this family is given in the following quotation from the committee report (p. 542):

> The Nitrobacteriaceae are clearly the most primitive of the Eubacteriales. Their power to live without complex organic substances would have made it possible, as Jensen points out, for them to flourish at a very early period in the world's history, and their simple structure is in harmony with the view that they represent the ancestral type of all other bacteria.

To accept this family, then, is really to endorse the theory that its members are modern representatives of the primordial bacteria.

Before endorsing that theory, however, it seems well to consider what evidence we have concerning the primordial types of bacteria. Such evidence cannot be obtained by paleontology, as when higher forms of life are concerned. What evidence we have from fossils as to the existence of bacteria in past ages, although scanty, is very interesting.[3] Recent deductions, however, such as those of Kligler (1917, p. 166) and of Osborn (1916, p. 292, and 1917, p. 86), as to the kinds of bacteria living in these early days, based upon the supposed protoplasmic structure of these fossilized organisms, will hardly be accepted by conservative bacteriologists.

Jensen's speculations as to the earliest forms of life are based upon chemical and physical considerations rather than upon paleontology and perhaps have a firmer foundation than those which rest upon Walcott's observations. Jensen concludes that the autotrophic bacteria were the first organisms on the earth, because they are the only known forms of life which can live upon inorganic matter without the action of sunlight, and

[3] See Walcott's evidence (1915) as to bacteria in Algonkian limestone, and Moodie's discussion (1916) of their existence in Mesozoic times.

he assumes that the primordial organisms must have lived in darkness and have depended upon inorganic matter for nutrition and energy. He overlooks the fact that if the earliest protoplasm possessed any such valuable property as the ability to utilize the chemical energy of inorganic material, it is strange that all but a very few of the organisms found today should have lost the power. He does not mention the idea that autotrophism may be a specialization developed by bacteria later on in the course of evolution; and yet the very organism which he selects as probably the most primitive of autotrophic bacteria, *B. methanicus* Söhngen (renamed *Methanomonas* by Jensen), derives its energy from methane liberated in swamps from decomposing organic matter. In other words, it gets its energy indirectly—through other organic agencies—from sunlight. Jensen's claim that under primordial conditions it utilized methane produced by volcanic action is a rather improbable assumption. It might be possible to advance more valid arguments in favor of the greater primitiveness of the nitrifying bacteria. Jensen does not deny this possibility; but he plainly believes that the most primitive organism is to be found among the autotrophic bacteria.

The committee on classification has apparently accepted this argument of Jensen's, as seen by a glance at the genera composing the family Nitrobacteriaceae. The first seven genera of Nitrobacteriaceae listed by the committee are the same and are even arranged in the same order as the seven genera placed by Jensen in his most primitive family, the Oxydobacteriaceae, except that for three of the genera the committee has recognized the validity of names prior to those of Jensen. It is evident that if the society adopts the committee report, it will be committing itself as favoring Jensen's arguments, and before doing this it is necessary to be sure that there are no other equally tenable theories as to primordial life.

As a matter of fact, other theories have been advanced which are by no means disproved by Jensen's arguments. Most important of them is the one that looks to the blue-green algae or to the little known phototrophic pigment-containing bacteria as the primordial organisms. These organisms, like

the autotrophic bacteria, use inorganic food, but they obtain their energy from sunlight instead of from chemical transformations. Jensen disregards this theory on the assumption that the primordial earth was dark; but Chamberlin's theory of the earth's origin has as much weight today as the nebular hypothesis, and according to his theory sunlight may have reached the earth's surface even in the earliest time.[4]

Another possibility not to be overlooked is that the earliest bacteria had energy-bearing carbon compounds at their disposal. These may have been formed by preceding life of still simpler nature, which is either extinct today or has escaped detection; or they may have been formed by inorganic agencies. Moore and Webster (1913) have shown that organic matter (formaldehyde) can be synthetized by the action of certain inorganic catalysts, which utilize sunlight energy, and have pointed out the significance of this fact as a possible explanation of the source of nutrition for the earliest life on the earth. If we assume that organic matter was synthesized by inorganic agencies before the existence of life, it is entirely unnecessary to look to phototrophic or autotrophic organisms as the original ancestors. The first organisms might have derived both food and energy from the simple carbon compounds then in existence.

The only justification for recognizing several genera of autotrophic bacteria is on the assumption that the few species we know are the sole survivors of primitive genera. This assumption, as just shown, is not the only reasonable hypothesis. A more conservative course than to accept the entire list of genera of Nitrobacteriaceae would be to recognize but one, or at the most, two genera of prototrophic bacteria. If two genera are to be recognized, one could include those forms capable of obtaining both their carbon and nitrogen from inorganic sources, and the other those requiring organic carbon but able to use elementary nitrogen. For these two genera, the names Nitrosomonas Winogradsky and Azotobacter Beijerinck would have to stand by priority.

[4] See Chamberlin (1916), p. 248. An earlier discussion of primordial conditions by Chamberlin and Chamberlin (1908) shows that life may perhaps have started under conditions not so very different from those of today.

There is no reason, indeed, for making a separate family of the phototrophic bacteria. As Jensen points out, most of them are rods with polar flagella, a fact which suggests that they are closely related to the heterotrophic *Pseudomonas* forms. They may easily be placed in the family Pseudomonadaceae recognized by the committee. It is true, as remarked by Buchanan (1917), that the spherical *Nitrosococcus* should not be separated from the rod-shaped *Nitrosomonas;* but this can easily be avoided without making a separate family for these organisms. One of Jensen's most valuable contributions to systematic bacteriology is in pointing out that the shape of cell or form of body is not a fundamental character and that there is not only a possibility but even a probability that transformations of cocci to rods and of rods to cocci may have taken place more than once in the course of development. This conception makes it entirely possible to place the autotrophic and possibly some simple heterotrophic cocci in the Pseudomonadaceae. It would merely require a definition of the family so worded as not to exclude all coccus forms.

The acceptance of Jensen's ideas as to the transformations of cocci into rods and rods into cocci does not force us to accept his theory that the most primitive forms were rods with polar flagella. In the natural course of development, a non-motile spherical organism would probably have preceded the motile, rod-shaped *Pseudomonas* type. As soon as a spherical organism becomes motile it tends to become elongated, a fact which undoubtedly explains the rarity of motile cocci. This thought gives another reason for hesitation before accepting the family Nitrobacteriaceae as the most primitive group of bacteria.

Family 2. Mycobacteriaceae. This family is placed by the committee immediately after the Nitrobacteriaceae. In this they follow Jensen. Jensen, however, uses this arrangement on the fallacious assumption that *Rhizobium* (the organism of legume nodules) has a polar flagellum, as formerly claimed by Harrison and Barlow (1907). On this assumption *Azotobacter* and *Rhizobium*, both with polar flagella and both able to utilize atmospheric nitrogen, formed natural stepping-stones between

the two groups. Jensen placed *Azotobacter* in one family and *Rhizobium* in the other. In their report as originally presented (Winslow, et al., 1917a), the committee on classification did the same; but in their complete published report (1917b) they have placed both *Azotobacter* and *Rhizobium* in the Nitrobacteriaceae.

This change may have been unintentional; in which case it is a very unfortunate mistake. DeRossi (1906), Zipfel (1911) and Kellerman (1912) and others have shown conclusively that the proper technic reveals peritrichous flagella on the legume organism.[5] The characterization of the genus given by the committee on page 553 of their report is therefore incorrect. That peritrichic organisms with granular structure and branching cells should be placed in the same family with the autotrophic bacteria can be justified only if physiology is allowed to override morphology entirely in establishing the classification. The committee distinctly state in regard to the Nitrobacteriaceae (p. 551) "When motile, with polar, never peritrichous, flagella."

The treatment of *Rhizobium* is important, because its relationships probably determine the position of the *Actinomyces* line. On the one hand its branching cells and granular structure show a striking resemblance to the tubercle organism and Actinomycetes, while on the other hand the motility of its vegetative rods helps to establish a connection with the true bacteria. Jensen accepted the old description of *Rhizobium* as a monotrichic rod and therefore placed the family of Actinomycetes in the Cephalotrichinae; but as the legume organism has now been definitely

[5] After finishing this paper the writers have noticed that Burrill and Hansen (Ill. Agr. Exp. Sta., Bul. 202. 1917) have apparently observed a single flagellum on this organism. As stated, however, by Hansen (who wrote the bulletin), this flagellum seems to be at a corner instead of at the pole, and the figures show it at times attached to the middle of the rod. This strongly suggests preparations that the writers have seen of known peritrichic organisms which, because of poor technic, reveal only a single flagellum on a rod. The work of such careful investigators as DeRossi, Kellerman, Zipfel and Prucha ought not to be dismissed with the few words granted to them by Hansen, while the still more recent work of Wilson (Cornell Agr. Exp. Sta., Bul. 386. 1917) apparently has not been seen by Hansen. The writers have seen microscopic preparations made by Kellerman which are very much better than his published photomicrographs and show peritrichous flagella without any question.

shown to have peritrichous flagella, the family is probably derived from the peritrichic stem, and does not belong near the Nitrobacteriaceae, where it has been placed by the committee. Buchanan, as previously mentioned, has wisely suggested the recognition of a separate order, calling it Actinomycetales. Perhaps the order might be subdivided into two families: Mycobacteriaceae for forms like the tubercle organism, Actinomycetaceae for the forms with true branched filaments.

All the genera in this group recognized by the committee probably have good standing, with the possible exception of *Nocardia* which as defined has no suitable type species.

Family 3. Pseudomonadaceae. This family has good standing, as does the genus *Pseudomonas,* also; but as already mentioned, the family might include the autotrophic as well as the heterotrophic forms. The definition of the family should be so worded as not to exclude all spherical or immotile forms that seem to be closely related to typical species. The committee has evidently made a mistake in saying "Flagella *single,* polar," thus leaving no place for the lophotrichic rods. Many of the fluorescent water bacteria which the committee plainly intends to put in this family have a tuft of four or more polar flagella.

Family 4. Spirillaceae. The arrangement of this family in two genera, as recognized by the committee, is apparently as satisfactory as any which can be suggested.

Family 5. Coccaceae. This family has been studied so thorroughly by Winslow and Winslow (1908) and its nomenclature given such careful scrutiny by Buchanan (1915) that there are no good reasons for criticising its genera as recognized by the committee.

The question arises whether this family is primitive or highly specialized. On the one hand the spherical form suggests primitiveness, while on the other the large number of animal parasites it contains plainly indicates recent development. As a matter of fact, the group seems to contain all gradations from soil and water forms that live on material of fairly simple composition up to parasites that are adapted to growth in living animals. Perhaps eventually we will have to accept Jensen's

theory that the spherical bacteria do not constitute a distinct evolutionary group but that the parasitic cocci are of an independent origin, more modern than the saprophytic ones.

The position of the genus *Streptococcus* is a puzzling matter. Jensen holds that rods gave rise to streptococci and that micrococci were derived from streptococci. This argument can be used only on the assumption just mentioned that the saprophytic cocci had an earlier independent origin from some other source. There are good reasons, moreover, for thinking that streptococci are more closely related to the chain-forming short rods occurring in milk than to the micrococci; while some bacteriologists place them near the diphtheroids (Mellon, 1917). Kligler (1917) separates them widely from the micrococci. The streptococci, in short, might easily be removed entirely from the Coccaceae; but because of the uncertainty of the whole matter, it seems best to be conservative and leave them, as the committee recommends in the same family with the other spherical bacteria.

Family 6. Bacteriaceae. There is some question just what this family should include. The committeee makes it include practically all peritrichic and non-motile, non-spore-forming rods, exclusive of the Bulgaricus group. The chief objection to this arrangement is that it tends to place in this family any member of the Pseudomonadaceae that has lost its power of motility. A non-motile, non-spore-forming rod cannot safely be assigned to either of these families until it has been studied sufficiently to make plain its relationships to other bacteria. A possible treatment of the non-motile, non-spore-forming rod-shaped bacteria would be to recognize an appendix to the families of Eubacteriales in which they may be placed until their relation to other better defined species is learned. The mycologists recognize a group which they call Fungi Imperfecti. Bacteriologists might equally well create a group of Bacteria Imperfecta.

The committee has placed four genera in the Bacteriaceae. The chief criticism against the selection of these genera is that too great weight has been placed upon pathogenicity. Two of them, *Hemophilus* and *Pasteurella,* include animal pathogens only; one, *Erwinia,* includes only plant pathogens; while the

third, into which apparently are to be dumped all peritrichic or non-motile, non-spore-forming saprophytes, is stated in the report to consist primarily of the colon-typhoid-dysentery group. It is particularly unfortunate that *B. coli* has been proposed as the type of this genus, since the group which centers around this organism is quite distinct from the majority of saprophytes belonging in this family. In this matter they claim to be accepting Jensen's emendation of the genus *Bacterium;* but they expand his emendation to include his *Denitrobacterium* and also the *Proteus* group, which Jensen named *Liquidobacterium,* and have actually, therefore, proposed a new emendation. If *B. coli* is accepted as the type of *Bacterium* it would be natural to use the generic name only for the colon-typhoid group, leaving no place for the large number of indefinite forms that the committee intends it to include.

Still more questionable is the genus *Erwinia.* This has been named as a new genus by the committee without a type species and therefore does not have a good standing. It is very doubtful, indeed, whether peritrichic plant parasites are sufficiently distinct from saprophytes to be put in a genus by themselves.

It seems strange that the committee has not recognized the genus *Proteus* Hauser, which is as distinct as several that they have recognized. Although this genus is primarily saprophytic, it has a fairly well defined type species, and is apparently distinct from the colon-typhoid group.

Family 7. Lactobacillaceae. There is very little reason for putting the Bulgaricus type of organisms in a family by themselves. They do differ in many respects from the Gram-negative rods—enough to justify the recognition of the genus *Lactobacillus,* but hardly enough to furnish a basis for the establishment of a new family. The very points of greatest distinction, granular structure, occurrence of bud-like branches, and failure to decolorize by the Gram method, are points which suggest a relationship to the diphtheria and tubercle organisms. So little is known about the *Lactobacillus* group, however, that there is scant justification for placing it in the Mycobacteriaceae, and the

most conservative procedure is to leave these forms in the Bacteriaceae.

Family 8. Bacillaceae. This family, for the spore-forming rods, has very good justification. The two genera, *Bacillus* and *Clostridium*, can probably be separated, but whether on the basis of relation to oxygen or of shape of the sporangium, the future must decide. Although relation to oxygen is a very important physiological distinction, it must be admitted that the selection of a physiological basis for the separation of these two genera is rather unsatisfactory. It places some of the polar-spored organisms in one genus, some in the other, and raises the question where to place facultative anaerobes like *B. mycoides* and *B. cereus*.

Type species. At the 1916 meeting of the society, the committee on classification proposed that American bacteriologists follow the international code of nomenclature adopted at the Vienna Botanical Congress. No opportunity had been given the society to study into the matter, and naturally the resolution was passed. It seems, however that the Vienna code does not recognize the principle of type species. The committee on classification (p. 531, footnote) states that the principle of type species "affects bacterial taxonomy less than other divisions of taxonomy." In this opinion they are decidedly open to criticism; for the very difficulty in defining bacterial genera and species accurately makes it imperative that no genus be recognized without a well-defined type. The fact that the society took action in favor of the Vienna code does not absolve bacteriologists from accepting this fundamental principle of nomenclature as it is recognized by zoölogists and by many botanists.

The committee has plainly considered type species unnecessary, for they have omitted to mention them for the following genera: *Nocardia*, *Pseudomonas*, *Spirillum*, *Albococcus*, *Rhodococcus*, and *Erwinia*. Some of these genera may easily be assigned type species (e.g., *Albococcus pyogenes* (Rosenbach) Winslow); but for others, such as *Nocardia*, none is available. It is especially unfortunate that the committee has neglected to name a type species for *Erwinia*, which they describe as a new genus.

CONCLUSIONS

In spite of all these criticisms of the report in matters of detail, it should be recognized that the committee has drawn up an admirable summary of the present status of systematic bacteriology. In matters of opinion, the names of the six members of the committee carry a great deal of weight. Even in matters of detail their classification is as satisfactory as could be drawn up by any other similar group of men. The report is, in reality, open to criticism mainly because of the extent of the undertaking which the society has placed upon the committee. No other committee on systematic biology appointed by a national or international society has ever undertaken such an ambitious task as a complete classification of any group of animals or plants. Other committees of this sort have done nothing further than to pass upon the validity of generic and specific names submitted to them, leaving it to individual initiative to propose new names, to classify and to define the groups.

Summing up the suggestions made above, the following classification of orders and families of Schizomycetes is obtained:

Order I. Eubacteriales. Cells minute; spherical, rod-shaped, or spiral, often occurring in chains, but never in branched or sheathed filaments.

Family 1. Pseudomonadaceae. Cells generally rod-shaped, though occasionally spherical. If motile, flagella occur at the pole. No endospores. Generally Gram-negative. Primarily water and soil forms. Sometimes able to assimilate inorganic nitrogen and to obtain energy from the oxidation of inorganic compounds. Often parasitic to plants, seldom to animals.

Family 2. Spirillaceae. Cells more or less spirally curved. If motile, flagella are polar.

Family 3. Coccaceae. Cells in their free conditions spherical; during division somewhat eliptical. Motility rare. Endospores absent. Often parasitic on animals.

Family 4. Bacteriaceae. Rod-shaped cells without endospores. Generally Gram-negative. Flagella when present peritrichic. Saprophytes, animal parasites and plant parasites.

Family 5. Bacillaceae. Rods producing endospores, usually Gram-positive. Flagella, when present, peritrichous. Primarily saprophytes secreting proteolytic enzymes. A few parasites.

Appendix. Bacteria Imperfecta. A temporary group to contain the non-motile, non-spore-forming rods, whose relationship to any of the above families cannot be shown.

Order II. Actinomycetales. Cells usually elongated, frequently filamentous and with a tendency to the development of branches.

Family 1. Mycobacteriaceae. Cells do not form true branched filaments. Frequently show swellings, clubbed or irregular shapes. Gram-positive. Frequently pathogenic. Aerobic.

Family 2. Actinomycetaceae. True filaments formed, developing into a definite branched mycelium. One-celled reproductive bodies formed by fragmentation. Aerobic and anaerobic. Occasionally pathogenic, but ordinarily living upon simple organic compounds.

Order III. Thiobacteriales. Cells free or united in elongated filaments. Cells typically containing either granules of free sulphur or bacterio-purpin or both, usually growing best in presence of hydrogen sulphide.

Order IV. Chlamydobacteriales. Cells normally in elongated filaments. Iron often present. Usually a well-marked sheath.

Order V. Myxobacteriales. Cells united during the vegetative state into a pseudoplasmodium which passes over into a highly developed cyst-producing resting stage.

Appendix. Spirochaetaceae. Spirilliform organisms, multiplying by transverse division. Frequently parasitic.

REFERENCES

BUCHANAN, R. E. 1915 Nomenclature of the Coccaceae. J. Inf. Dis. **17**, 528–541.

BUCHANAN, R. E. 1917 Studies in the nomenclature and classification of the bacteria. J. Bact, **1**, 591–596, **2**, 155–164, 347–350.

CHAMBERLIN, T. C. 1916 The origin of the earth. Chicago.

CHAMBERLIN, T. C. and CHAMBERLIN, R. T. 1908 Early terrestrial conditions that may have favored organic synthesis. Science, n. ser. **28**, 897–911.

CONN, H. J. 1917 Actinomycetes in soil. N. Y. Agr. Exp. Sta., Tech. Bul. **60**.

DeRossi, G. 1906 Ueber die Mikroorganismen welche die Wurzelknölchen der Leguminosen erzeugen. Centbl. Bakt., II Abt., **18**, 289–314, 481–489.

Harrison, F. C., and Barlow, B. 1907 The nodule organism of the Leguminosae. Centbl. Bakt., II Abt., **19**, 264–272, 426–441.

Jensen, O. 1909 Die Hauptlinien des natürlichen Bakteriensystems. Centbl Bakt., II Abt., **22**, 305–346.

Kellerman, K. F. 1912 The present status of soil inoculation. Centbl. Bakt., II Abt., **34**, 42–50.

Kligler, I. J. 1917 The evolution and relationships of the great groups of bacteria. J. Bact. **2**, 165–176.

Lachner-Sandoval, V. 1898 Ueber Strahlenpilze. Strassburg Univ., Inaug. Dissert.

Mellon, R. R. 1917 A study of the diphtheroid group of organisms with special reference to its relation to the streptococci. J. Bact. **2**, 81–108, 269–308, 447–500.

Moodie, R. L. 1916 Mesozoic pathology and bacteriology. Science, n. ser., **43**, 425–426.

Moore, B., and Webster, T. A. 1913 Synthesis by sunlight in relationship to the origin of life. Roy. Soc., Proc. B., **87**, 163–176.

Neukirch, H. 1903 Ueber Strahlenpilze II. Strassburg Univ., Inaug. dissert.

Osborn, H. F. 1916 The origin and evolution of life upon the earth. III. Sci. Monthly, **3**, 289–307.

Osborn, H. F. 1917 The origin and evolution of life. New York.

Walcott, C. D. 1915 Discovery of Algonkian bacteria. Proc. Nat. Acad. Sci., **1**, 256–257.

Winslow, C.-E. A., and Winslow, A. R. 1908 The systematic relationships of the Coccaceae. New York.

Winslow, C.-E. A., et al. 1917 a. The families and genera of bacteria. Abstr. of Bact. **1**, 25.

Winslow, C.-E. A., et al. 1917 b. The families and genera of the bacteria. Preliminary report of the committee of the Society of American Bacteriologists on characterization and classification of bacterial types. J. Bact. **2**, 505–566.

Zipfel, H. 1911 Beitrage zur Morphologie und Biologie der Knollchenbakterien der Leguminosen. Centbl. Bakt., II Abt., **32**, 97–137.

STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

IX. THE SUBGROUPS AND GENERA OF THE THIOBACTERIALES

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Order IV. **Thiobacteriales** Ordo nov.

Cells various, typically containing either granules of free sulphur, or bacteriopurpurin, or both, usually growing best in the presence of hydrogen sulphide. The cells are plant-like, not protozoan-like, not producing a pseudoplasmodium or a highly developed encysted resting stage. Spores are rarely or never formed.

Classification within this group is in a very unsatisfactory and very superficial state. Few investigators have studied these forms, and most of the work is old, and in need of careful revision. Undoubtedly many of the genera are to be regarded as growth forms, merely.

The following names have been applied to families, subfamilies, tribes and subtribes.

Chromatiaceae Migula, 1900, p. 1047
Amoebobacterieae De Toni and Trevisan, 1889, p. 1043
Beggiatoaceae Migula, 1895, p. 41
Amoebobacteriaceae Migula, 1900, p. 1045
Thiocapsaceae Migula, 1900, p. 1042
Thiopediaceae Migula, 1900, p. 1044
Rhodobacteriaceae Migula, 1900, p. 1042
Lamprocystaceae Migula, 1900, p. 1043
Athiorhodaceae Molisch, 1907, p. 28
Thiobacteriaceae Jensen, 1909, p. 303

The order Thiobacteriales may be divided into families as follows:

Key to the families of Thiobacteriales

A. Cells containing sulphur granules (or in one species possibly oxalate crystals), but no bacteriopurpurin.
   1. Unicellular, motile forms. Not filamentous. Family I. *Achromatiaceæ*
   2. Filamentous forms..........................Family II. *Beggiatoaceae*

B. Cells containing bacteriopurpurin with or without sulphur granules. Family III. *Rhodobacteriaceæ*

Family I. **Achromatiaceae** Fam. nov.

Unicellular, large, motile (by means of flagella?) cells containing granules of sulphur (or in one form possibly oxalate), but no bacteriopurpurin.

The following key will separate the genera recognized.

Key to the genera of Achromatiaceae

A. Cells spherical or ellipsoidal
   1. Cells ellipsoidal (spherical when newly divided). Cells containing granules of calcium oxalate (perhaps sulfur). Genus I. *Achromatium*
   2. Cells spherical, with sulphur granules in a central vacuole. Genus 2. *Thiophysa*

B. Cells longer, very large (42 to 86μ) with peritrichous flagella. Genus. 3. *Hillhousia*

Genus 1. **Achromatium** Schewiakoff 1893

Synonyms:

Modderula Frenzel, 1897, p. 901

Cells large, nearly spherical in newly divided cells to ellipsoidal, 15 to *43 by 9 to 22μ. Cells closely packed with large granules, at first interpreted as sulphur, but later interpreted as calcium oxalate. When granules are dissolved, cells show coarse alveolar structure. Cells are motile, flagella not demonstrated. Cell division resembles the constriction of flagellates rather than the fission characteristic of bacteria.*

The type species is *Achromatium oxaliferum* Schewiakoff. The organism occurs in the slime at the bottom of the rivers, in the so called "Modder."

Genus 2. **Thiophysa** Hinze, 1903, p. 310

Spherical cells laden with sulphur. The protoplasmic layer surrounds a large central vacuole. Cell nucleus not recognized. Flagella lacking. Cells elongate before division, divide to biscuit shaped cells. Cells 7 to 18μ in diameter.

The type species *Thiophysa volutans* Hinze was secured from the Bay of Naples.

Genus 3. **Hillhousia** West and Griffiths, 1909, p. 398

Cells very large, 42 to 86 by 20 to 33μ. motile by means of peritrichous flagella. Cells packed with large globules of oily amorphous sulphur.

The type species is *Hillhousia mirabilis* West and Griffiths.

Family II. **Beggiatoaceae** Migula, 1895, p. 41

Filamentous bacteria, usually showing an oscillating motion similar to Oscillatoria. Cells contain sulphur granules. Spore formation and conidia unknown.

The genera of the family *Beggiatoaceae* may be differentiated by means of the following key:

Key to the genera of Beggiatoaceae

A. Filament non-motile, with a contrast to base and tip, attached.
Genus 1. *Thiothrix*

B. Filaments motile (oscillating) not attached, no differentiation into tip and base.
   1. Filaments not in bundles nor surrounded by a gelatinous sheath.
   Genus 2. *Beggiatoa*
   2. Filaments in bundles, surrounded by a gelatinous sheath.
   Genus. 3. *Thioploca*

Genus 1. **Thiothrix** Winogradsky, 1888, p. 39

Filament non-motile, segmented, a definite differentiation into base and lip, attached, usually filled with sulphur granules. The threads produce rod shaped conidia at their ends. These conidia are self motile by means of a slow creeping motion, attach themselves and develop into new threads. The habitat is hot sulphur springs.

The type species is *Thiothrix nivea.* (Rabenhorst) Winogradsky.

Genus 2. **Beggiatoa** Trevisan, 1842, p. 76

Threads sheathless, formed of flat discoidal cells, not attached. Multiplication by transverse splitting of the threads. Showing an undulating motion, creeping. Cells contain globules of sulphur. Usually in hot sulphur springs.

The type species is *Beggiatoa alba* (Vaucher) Trevisan.

Genus 3. **Thioploca** Lauterborn, 1907, p. 238

Filaments Beggiatoa-like, with numerous sulphur granules, motile, lying parallel in considerable numbers, or united in bundles enclosed in a colorless layer of gelatin.

The type species, *Thioploca schmidlei* Lauterborn has filaments 5 to 9μ thick, and gelatinous sheath 50 to 160μ thick. From the ocean bed.

Family III. **Rhodobacteriaceae** Migula, 1900, p. 1042

Synonym:

Rhodobacteria Molisch, 1907, p. 27

Cells of various types, not filamentous, containing bacterio-purpurin with or without sulphur granules also.

Two subfamilies may be separated by the following key:

Key to the subfamilies of Rhodobacteriaceae

A. Cells containing sulphur granules..............Subfamily I. *Chromatioideae*
B. Cells without sulphur granules...........Subfamily II. *Rhodobacterioideae*

Subfamily I. **Chromatioideae** Nom. nov.

Synonyms:

Thiorhodaceae Molisch, 1907, p. 28

Cells not filamentous, containing both sulphur granules and bacteriopurpurin.

The following names have been used for genera in this group:

Erythroconis Oersted, 1842, p. 552
Chromatium Perty, 1852, p. 179
Clathrocystis Henfrey, 1856, p. 53
Rhabdomonas, Cohn, 1875, p. 167

Cohnia Winter, 1884, p. 48
Lamprocystis Schroeter, 1886, p. 151
Lampropedia Schroeter, 1886, p. 151
Mycothece Hansgirg, 1888, p. 266
Amoebobacter Winogradsky, 1888, p. 71
Thiocapsa Winogradsky, 1888, p. 84
Thiocystis Winogradsky, 1888, p. 60
Thiodictyon Winogradsky, 1888, p. 80
Thiopedia Winogradsky, 1888, p. 85
Thiopolycoccus Winogradsky, 1888, p. 79
Thiosarcina Winogradsky, 1888, p. 104
Thiospirillum Winogradsky, 1888, p. 104
Thiothece Winogradsky, 1888, p. 82
Cenomesia De Toni and Trevisan, 1889, p. 1039
Thiosphaerion Miyoshi, 1897, p. 170
Thiosphaera Miyoshi, 1897, p. 170
Rhodocapsa Molisch, 1906, p. 223
Rhodothece Molisch, 1906, p. 223
Amoebomonas Jensen, 1909, p. 338
Thioderma Miyoshi, 1897, p. 170
Rhabdochromatium Winogradsky, 1888, p. 100

Of these names *Clathrocystis* and *Erythroconis* are algal genera to which certain of the sulphur bacteria have at different times been assigned.

Following is a key to the tribes of the *Chromatioideae* which may be recognized largely from the descriptions of Winogradsky.

Key to the tribes of Chromatioideae

A. Cells united, at least during a part of the life history, into families.
    I. Cell division such that masses of cells, not merely plates, are formed.
        a. Cell division in three directions of space.....Tribe I. *Thiocapseae*
        b. Cell division first in three, then in two directions of space.
            Tribe II. *Lamprocysteae*
    II. Cell division in two planes, forming plates of cells.
            Tribe III. *Thiopedieae*
    III. Cell division in one plane................Tribe IV. *Amoebobacterieae*
B. Cells free, capable of swarming at any time........Tribe V. *Chromatieae*

Tribe I. **Thiocapseae** Trib. nov.

Synonyms:

Thiocapsaceae Migula, 1900, p. 1042

Bacteria containing both sulphur granules and bacteriopurpurin. Cells divide in three directions of space, united into families.

Key to the genera of the Thiocapseae

A. Cells capable of swarming.
   I. Families small, compact, enclosed singly or several together in a cyst..Genus I. *Thiocystis*
   II. Cells large, 7 to 8μ loosely bound by gelatin into loose families Genus II. *Thiosphaera*
   III. Cells small, united into solid, spherical families. Genus III. *Thiosphaerion*

B. Cells not capable of swarming.
   I. Spherical cells spread out upon the substratum in flat families, loosely enveloped in a common gelatin................Genus IV. *Thiocapsa*
   II. Arranged in regular packets like *Sarcina*......Genus V. *Thiosarcina*

Genus I. **Thiocystis** Winogradsky, 1888, p. 60

Usually 4 to 20 or 30 cells massed into small, compact families, enveloped singly or several together in a gelatinous cyst, capable of swarming. When the families have reached a definite size they escape from the gelatinous cyst, the latter either swelling and softening uniformly or at some particular spot. The escaped cells either pass into the swarm stage or unite into a larger fused complex of families. Cells are light colored, single cells almost colorless. In masses the cells show a beautiful violet or red violet color. The cells are frequently quite filled with sulphur granules.

The type species is *Thiocystis violacea* Winogradsky.

Genus II. **Thiosphaera** Miyoshi, 1897, p. 170

Cells spherical-ellipsoidal, relatively large (7 to 8μ) light violet in color, bound into loose families by a colorless gelatin. Capable of swarming. Sulphur inclusions relatively abundant.

The type species is *Thiosphaera gelatinosa* Miyoshi.

Genus III. **Thiosphaerion** Miyoshi, 1897, p. 170

Cells spherical-ellipsoidal, small (1.8 to 2.5μ violet in color, with delicate sulphur inclusions. United by means of gelatin into solid spherical families. Capable of swarming.

The type species is *Thiosphaerion violaceum* Miyoshi.

Genus IV. **Thiocapsa** Winogradsky, 1888, p. 84

Cell families resembling in grouping and multiplication the cells of the algal genus Aphanocapsa. Cell division occurs in all directions of space, the cells are spherical, with thick confluent membranes, which unite to form a structureless, gelatinous layer. The cells are of a bright rose red color and contain numerous sulphur granules. The cells do not swarm.

The type species is *Thiocapsa roseo-persicina* Winogradsky.

Genus V. **Thiosarcina** Winogradsky, 1888, p. 104

Synonym:

Rhodosarcina Jensen, 1909, p. 334

Non-swarming cells arranged in packet shaped families, corresponding to the genus Sarcina. Cells red, with sulphur granules.

The type species is *Thiosarcina rosea* (Schroeter) Winogradsky.

Tribe II. **Lamprocysteae** Trib. nov.

Synonyms:

Lamprocystaceae Migula, 1900, p. 1043

Cells united into families in which division of the cells occur first in three planes, then in two.

The single genus of this tribe is *Lamprocystis*.

Genus I. **Lamprocystis** Schroeter, 1886, p. 151

Synonyms:

Clathrocystis Cohn, 1875
not *Clathrocystis* Henfrey, 1856, p. 53
Cohnia Winter, 1884, p. 48
not *Cohnia* Kunth 1850
not *Cohnia* Reichenbach 1852
Cenomesia? De Toni and Trevisan, 1889, p. 1039

Cells ellipsoidal, dividing at first in three planes to form spherical cell masses, later in two planes, forming hollow sacks in which the cells lie embedded in a layer in the walls, finally the membrane ruptures, and the whole mass becomes net like, much as in the algal genus Clathrocystis. Usually colored intensely violet. Small sulphur granules present. Capable of swarming.

The type species is *Lamprocystis roseo-persicina* (Cohn) Schroeter.

Tribe III. **Thiopedieae** Trib. nov.

Synonyms:
Thiopediaceae Migula, 1900, p. 1044

Sulphur bacteria in which the cells are united into families, and cell division is in two directions of space, resulting in the development of plates of cells.

The two genera may be differentiated by the following key:

Key to the genera of Thiopedieae

A. Cells occurring regularly in fours .... Genus I. *Lampropedia*
B. Cells occurring in a film or membrane, not regularly disposed in tetrads. Genus II. *Thioderma*

Genus I. **Lampropedia** Schroeter, 1886, p. 151

Synonyms:
Erythroconis? Oersted, 1842, p. 6
Thiopedia Winogradsky, 1888, p. 85

Cells united into tetrads, forming flat tubular masses. Contain sulphur granules and bacteriopurpurin.

The type species is *Lampropedia hyalina* (Kuetzing) Schroeter.

Genus II. **Thioderma** Miyoshi, 1897, p. 170

Cells spheroidal, light rose in color, containing small, inconspicuous, sulphur granules. United into thin purplish membrane,

The type species is *Thioderma roseum* Miyoshi.

Tribe IV. **Amoebobacterieae** Trib. nov.

Synonyms:

Amoebobacteriaceae Migula, 1900, p. 1045

Sulphur bacteria in which the cells are united into families. Cell division occurring only in one direction of space.

Key to the genera of Amoebobacterieae

I. Cells connected by plasma threads, families amoeboid motile.
Genus I. *Amoebobacter*

II. Cells not as I.
- A. Cells arranged in a net, united by their ends.....Genus II. *Thiodictyon*
- B. Cells not arranged in a net.
  1. Capable of swarming. Cells loosely aggregated in gelatin.
  Genus III. *Thiothece*
  2. Non-motile. Cells closely appressed into a colony.
  Genus IV. *Thiopolycoccus*

Genus I. **Amoebobacter** Winogradsky, 1888, p. 71

Cells connected by plasma threads. Families amoeboid motile. The cell families slowly change form, the cells drawing together into a heap or spreading out widely, thus bringing about a change in the shape of the whole family. In a resting condition a common gelatin is extruded, the surface becomes a firm membrane.

The type species is *Amoebobacter roseus* Winogradsky.

Genus II. **Thiodictyon** Winogradsky, 1888, p. 80

Synonym:

Rhododictyon Jensen, 1909, p. 334

Cells rod-shaped or spindle-shaped, with sharply pointed ends, united into a net. The compact mass of rods finally assumes an appearance like that of Hydrodictyon. Slight violet color.

The type species is *Thiodictyon elegans* Winogradsky.

Genus III. **Thiothece** Winogradsky, 1888, p. 82

Cells spherical, in families enclosed in a thick gelatinous cyst. Cells capable of swarming and very loosely embedded in a common gelatin. When the swarm stage supervenes, the cells lie more

loosely, the gelatin is swollen, and the cells swarm out singly and rather irregularly.

The type species is *Thiothece gelatinosa* Winogradsky.

Genus IV. **Thiopolycoccus** Winogradsky, 1888, p. 79

Families solid, non-motile, consisting of small cells closely appressed. Multiplication of the colonies by the breaking up of the surface into numerous short shreds and lobes which continue to split up into smaller heaps. Cells red.

The type species is *Thiopolycoccus ruber* Winogradsky.

Tribe V. **Chromatieae** Trib. nov.

Synonym:

Chromatiaceae Migula, 1900, p. 1047

Sulphur bacteria in which the cells are not united into families, but free, and capable of swarming at any time.

The genera of the tribe *Chromatieae* may be differentiated by the following key:

Key to the genera of Chromatieae

A. Cells motile by means of polar flagella. Elongated.
  I. Cells not spiral.
    a. Cells cylindric............................Genus I. *Chromatium*
    b. Cells with tendency to spindle shape.....Genus II. *Rhabdomonas*
  II. Cells spiral.................................Genus III. *Thiospirillum*
B. Cells spherical, or little elongate, non motile.
  I. Cells not encapsulated........................Genus IV. *Rhodocapsa*
  II. Cells encapsulated in pairs.....................Genus V. *Rhodothece*

Genus I. **Chromatium** Perty, 1852

Synonym:

Rhodomonas Jensen, 1909, p. 334

Cells cylindric-elliptical or relatively thick cylindrical. Cell contents red, containing dark sulphur granules. Cells somewhat variable in shape, straight, more or less bent, short cells ovoid and longer forms more cylindrical. Motile by means of polar flagella.

The type species is *Chromatium okenii* Perty.

Genus II. **Rhabdomonas** Cohn, 1875, p. 167

Synonyms:

Mantegazzaea Trevisan, 1879, p. 137 in part
Rhabdochromatium Winogradsky, 1888, p. 150

Differentiated from Chromatium by the elongated rod-shaped or spindle-shaped cells. Cells red, with sulphur granules, polar flagella.

The type species is *Rhabdomonas rosea* Cohn.

Genus III. **Thiospirillum** Winogradsky, 1888, p. 104

Synonym:

Ophidomonas? Ehrenberg.

Spiral motile bacteria containing sulphur granules and bacteriopurpurin.

The type species is *Thiospirillum sanguineum* (Ehrenberg) Winogradsky.

Genus IV. **Rhodocapsa** Molisch, 1906, p. 223

Cells spherical, free (not united into families) not capable of swarming (non-motile). In mass the organisms are cherry red. Contain sulphur granules.

The type species is *Rhodocapsa suspensa* Molisch.

Genus V. **Rhodothece** Molisch, 1906, p. 223

Cells usually spherical and in pairs, each surrounded by a spherical or an ellipsoidal capsule. Non-motile. Cells not united into families. Cells contain bacteriopurpurin and sulphur granules.

The type species is *Rhodothece pendens* Molisch.

Subfamily II. **Rhodobacterioideae** Subfam. nov.

Synonym:

Athiorhodaceae Molisch, 1907, p. 28

Cells not filamentous, containing bacteriopurpurin but not granules of sulphur.

The genera of this subfamily have all been described by Molisch. They may be differentiated by the following key:

Key to the genera of Rhodobacteroideae

I. Cells united into families.
  A. Cells rod shaped, many embedded in the same slimy capsule. Genus I. *Rhodocystis*
  B. Cells spherical or short rods.
    1. In chains, each chain surrounded by a capsule. Genus II. *Rhodonostoc*
    2. Cells free..............................Genus III. *Rhodosphaera*
  C. Cells free and elongate.
    1. Cells not bent.
      a. Non-motile..........................Genus IV. *Rhodobacterium*
      b. Motile..............................Genus V. *Rhodobacillus*
    2. Cells bent or curved.
      a. Cells short, comma shaped, with single polar flagellum Genus VI. *Rhodovibrio*
      b. Cells spiral, with polar flagella..Genus VII. *Rhodospirillum*

Genus I. **Rhodocystis** Molisch, 1907, p. 22

Cells rod-shaped, dividing in only one plane embedded in a common slimy capsule.

The type species is *Rhodocystis gelatinosa* Molisch.

Genus II. **Rhodonostoc** Molisch, 1907, p. 23

Cells spherical or short rods, in rosary like chains, and embedded in a common gelatinous capsule.

The type species is *Rhodonostoc capsulatus* Molisch

Genus III. **Rhodosphaera** gen. nov.

Synonym:
Rhodococcus Molisch, 1907, p. 20
not *Rhodococcus* Zopf, 1891, p. 28

Cells spherical, non-motile, free not united into families.

The type species is *Rhodosphaera capsulatus* (Molisch) Buchanan.

Genus IV. **Rhodobacterium** Molisch, 1907, p. 16

Rod shaped cells, non-motile, not united into families.

The type species is *Rhodobacterium capsulatum* Molisch.

Genus V. **Rhodobacillus** Molisch, 1907, p. 14

Rod shaped cells, solitary usually, motile.
The type species is *Rhodobacillus palustris* Molisch.

Genus VI. **Rhodovibrio** Molisch, 1907, p. 21.

Cells short, comma shaped, free, actively motile by means of a single terminal flagellum.
The type species is *Rhodovibrio parvus* Molisch.

Genus VII. **Rhodospirillum** Molisch, 1907, p. 24

Cells spiral, actively motile by means of polar flagella.
Rhodospirillum rubrum (Esmarch) Molisch is the type species.

REFERENCES

De Toni, J. B., and Trevisan, V. 1889 Schizomycetaceae Naeg. in Saccardo's Sylloge Fungorum., **8**, 923–1087.

Frenzel 1897 Neue oder wenig bekannten Süsswasserprotisten. Biol. Cent., **17**, 801–808.

Hansgirg, Anton 1888 Beiträge zur Kenntniss der Kellerbakterien. Oesterr botan. Zeitschr. **38**, 227–230, 260–267.

Henfrey, Arthur 1856 Notes on some fresh water confervoid Algae new to Britain. Trans. Micr. Soc. of London, new series, **4**, 49–54.

Hinze, G. 1903 *Thiophysa volutans*, ein neues Schwefelbakterium. Ber. d. d. Bot. Ges., **21**, 309.

Jensen, Orla 1909 Die Hauptlinien des natürlichen Bakterien-systems. Cent. f. Bakt. Par. u. Inf., Abt. 2, **22**, 305–346.

Lauterborn, Robert 1907 Ein neue Gattung der Schwefelbakterien. (Thioploca Schmidlei) nov. gen. nov. spec. Ber. d. d. bot. Gesellschaft., **25**, 238–242.

Migula, W. 1895 Schizomycetes, in Engler and Prantl. Natürlichen Pflanzenfamilien, p. 20.

Migula, W. 1900 System der Bakterien, **2**.

Miyoshi, Manabu 1897 Studien über die Schwefelrasenbildung und die Schwefelbakterien der Thermen von Yumoto bei Nikko, Journal of the College of Science, Imperial University of Japan, **10**, 143–170.

Molisch, Hans 1906 Zeiw neue Purpurbakterien mit Schwefelkörperchen. Botan. Zeitg., Abt. 1, Orig. **64**, 223–232.

Molisch, Hans 1907 Die Purpurbakterien. Jena.

Oersted 1840–41 Beretnung om en Excursion. Naturh. Tiddsskrift II f.

Perty 1852 Zur Kenntniss kleinster Lebensformen. Bern.

Schewiakoff, W. 1893 Ueber einen neuen bacterienähnlichen Organismus des Süsswassers. Heidelberg.

SCHROETER, J. 1886 Die Pilze, in Cohns Kryptogamen Flora von Schlesien.
TREVISAN, V. 1842 Prospetto della Flora Euganea.
TREVISAN, V. 1789 Prime linee d'introduzione allo studio dei Batterj. italiani. Rendiconti. Reale Istituto Lombardo di Scienze e Lettere IV. Series 2, **12**, 133–151.
WEST, G. S., AND GRIFFITHS, B. M. 1909 Hillhousia mirabilis, a giant sulfur Bacterium. Proc. Roy. Soc. London. Biol. Sci., **81**, 398–399.
WINOGRADSKY, SERGIUS 1888 Zur Morphologie und Physiologie der Schwefelbacterien. Beiträge zur Morphologie und Physiologie der Bakterien. Heft. 1.
WINTER, GEORG 1884 Die Pilze, in Rabenhorst's Kryptogamen Flora. Ed. 2, vol. 1.
ZOPF, W. 1891 Ueber Auscheidung von Fettfarbstoffen (Lipochromen) seitens gewisser Spaltipilze. Ber. d. d. Bot. Gesellschaft. **9**, 28.

STUDIES ON PROTEOLYTIC ACTIVITIES OF SOIL MICROÖRGANISMS, WITH SPECIAL REFERENCE TO FUNGI[1]

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INTRODUCTION

It has long been recognized that when organic matter is applied to the soil it must be broken down into certain simple compounds before it can be assimilated by the roots of the plants and built up again into living tissues. This holds true, particularly, with nitrogenous compounds, such as the different proteins found in the plant residues, animal manures, and other protein substances applied to the soil. The plant is unable to assimilate the nitrogen from the complex protein bodies, and the latter must first be decomposed by means of other agencies before the assimilation of the nitrogen by the plant can take place. It is not known whether the organic and mineral acids found in the soil or those possibly excreted by the roots of the plants play any part in the hydrolysis of these proteins. But it is known that the microorganisms of the soil are able to decompose the proteins and liberate the nitrogen, which is enclosed in the complex protein molecule, in a simple form, which, either at once or after undergoing transformation due to the action of other bacteria, is easily assimilable by the roots of higher plants.

A great deal of attention has been paid by the students of soil fertility to this process of decomposition, which the organic matter undergoes in the soil; it would take up too much space to

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enumerate all the work that has been done along these lines, particularly the so-called "ammonification" studies. The fact is almost decided upon that the microörganisms decompose the organic matter in the soil, liberating ammonia, which is oxidized by other groups of organisms to nitrites, and then to nitrates, in which form it is utilized by higher plants. Several investigations seem to point to the fact that, in the absence of nitrates, other compounds, such as certain amino-acids, as shown by Skinner (1912a) and Brigham (1917), creatinine, studied by Skinner (1912b) and Brigham (1917), amides and ammonium salts, as shown by Hutchinson and Miller (1912), and other organic compounds (Brigham, 1917) can also be utilized by plants.

Very little work has been done on the process of decomposition of proteins as such, taking into consideration the intermediate steps and finding out whether the ammonia and other substances formed are merely products of metabolism or are final decomposition products. The organisms themselves must be studied, so as to learn by what biochemical processes the work is accomplished and what ends are attained, and only thus shall we be able to learn how to utilize the microörganisms for our needs in the economy of the soil and in other lines of human endeavor.

HISTORICAL

The work of Fisher and his followers, having firmly established the general structure of the protein molecule, has given a great impetus to the study of the hydrolysis and the synthesis of proteins by chemical and biological means. A great many investigations have been made of the action of bacteria and molds on proteins. It has been conceded that a great deal of this work is done by enzymes, but most of the data at hand are merely qualitative in nature, and few investigators have obtained definite quantitative information concerning the action of microorganisms or their enzymes upon protein compounds.

It has not been decided as yet, what nitrogen compounds form the *Bausteine* for these organisms. Czapek (1902) and others have shown that amino-acids form a much better source of nitro-

gen for fungi than other nitrogen compounds. Hence he argued that since the fungi must build their own proteins out of the ammonium compounds and nitrates through the amino-acid-stage, if the latter is offered to the organisms as a source of nitrogen, it is assimilated as such and the organism is spared unnecessary loss of energy, which it would have to spend in the building up of these amino-acids. Abderhalden and Rona (1905) have shown that when KNO_3, glutamic acid, and glycocoll are offered to the organisms as sources of nitrogen, the proteins that are built up will contain the same amino-acids in all cases, namely glycocoll, alanine, leucine, glutamic and aspartic acids. Hence they argued that the nitrogen building stones for these organisms must be ammonia; all the nitrogen compounds must be first split into ammonia, and out of this form of nitrogen all the proteins are built up, going through the amino-acid stage. Hagem (1910) supported the claim of Abderhalden and Rona (1905) by showing that the oxy-acids of ammonia are utilized just as well by the fungi as amino-acids; therefore he thought that in the case of amino-acids, both the nitrogen and carbon are utilized by the organism: the amino-acid is first split to ammonia and oxy-acid; the ammonia is utilized for the nitrogen needs, and the oxy-acids form a ready radical for the building up of the numerous amino-acids which go to make up the fungus protein. The oxv-acids of ammonia behave in a similar way, therefore these compounds offer a better source of food to the organism than other nitrogen compounds.

Butkewitsch (1903) identified tyrosine and leucine among the amino-acids produced by the action of *Aspergillus niger* and *Mucor stolonifer* upon peptone. Rettger and his associates (1916) have shown that bacteria are unable to attack or bring about the decomposition of proteins without the aid of enzymes or other proteolytic agents; this applies not only to the more complex proteins, like egg-albumen, but in all probability to albumoses and peptones as well. A wider range of ability to attack nitrogenous substances is observed for molds, as can be seen from the work of Czapek (1902) Emmerling (1902), Brenner (1914), and others. Sears (1916) has shown that peptone cul-

tures of most bacteria give fluctuating concentrations of amino-acids, as measured by the Van Slyke apparatus; these bodies are formed and broken down continuously by the organisms, with the exception of a few strongly proteolytic bacteria; while the ammonia gradually accumulates in the medium, the amino-acid content fluctuates daily and gradually decreases in most instances. Itano (1916) using the formol-titration method of Sörensen has shown that *B. subtilis* produced a gradual increase of formol-titrating nitrogen for a period of 240 hours. The greatest proteolysis took place toward the optimum hydrogen-ion concentration; therefore he suggested the probability that the enzyme is tryptic-like in nature; on filtering the bacterial cultures, he obtained no splitting of the peptone with the filtrate, but did obtain it with the bacterial mass.

These proteolytic changes are very important in the study of soil fertility. Schreiner and Shorey (1910) isolated from the soil, among numerous other organic compounds, the amino-acids histidine, arginine, and lysine, using a weak alkali extract of soil. That these amino-acids can be utilized by the plants as well as nitrates is seen from the work of Skinner (1912a), who has shown that histidine and arginine have a beneficial effect upon plant growth and can replace nitrates.

A great deal of work has been done on the production of ammonia from different organic compounds through the action of microörganisms, but no attempt will be made to review that work in this paper, because the study of "ammonification" is thought to be of doubtful importance, as a factor in the fertility of the soil or activities of microörganisms, when the other factors controlling these activities are not taken into consideration.

EXPERIMENTAL

Organisms used

Aspergillus niger van Tieghem. This organism was isolated on the College Farm, at New Brunswick, N. J., from a Sassafras sandy loam that was under orchard for over twenty years. Methods of isolation and the description of soils for this as well

as for the following organisms can be found in another place (Waksman, 1916).

Aspergillus ochraceus Wilhelm. This organism was isolated from a New Jersey Alloway clay soil, which was under timothy at the time of isolation.

Aspergillus fuscus Schiemann.

Aspergillus clavatus Desmasières.

Citromyces glaber Wehmer.

Penicillium chrysogenum Thom.

Actinomyces n. sp. *penicilloides* Waksman and Curtis. A full description of this organism will appear soon.

Actinomyces violaceus-ruber Waksman and Curtis.

Actinomyces diastaticus (Krainsky) Waksman and Curtis.

Bacterium mycoides Flügge, obtained from Dr. C. B. Lipman, of the University of California. All the other organisms were isolated by the writer from different soils.

Methods used

The organisms were grown on Czapek's solution composed as follows: $NaNO_3$ 2 grams, K_2HPO_4 1 gram, KCl 0.5 gram, $MgSO_4$ 0.5 gram, $FeSO_4$ 0.01 gram, cane sugar 30 grams, distilled water 1000 cc. Another medium was also used, by substituting peptone or casein for the $NaNO_3$, or for both the nitrate and cane sugar. The media were distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, which were plugged and sterilized for fifteen minutes at 15 pounds pressure; they were then inoculated with the proper organisms and incubated at 28°C. At the end of the proper incubation period, the cultures were filtered through filter paper and the filtrates used for the determination of ammonia and amino nitrogen. The aeration method of Folin (1902) was used for ammonia determinations, continuing the aeration for three hours, since a shorter period of time did not liberate all the ammonia. In certain determinations, after all the ammonia had been removed by the use of Na_2CO_3, 1 gram NaOH and 10 grams NaCl were added and aeration continued for one hour more. The use of NaOH and NaCl was recom-

mended by Steel (1910) and others as a general procedure for the determination of ammonia, so as to eliminate the formation of triple phosphate crystals which are formed on adding Na_2CO_3. But Potter and Snyder (1915) have pointed out the fact that the use of NaOH will cause a great deal of the amide nitrogen to be given off as ammonia. After the ammonia was completely removed, the solution was neutralized with acetic acid, and a

TABLE 1

The formation of amino nitrogen from casein and peptone by microörganisms

Milligrams of NH_2-N per 100 cc. of solution

PERIOD OF INCUBATION	ORGANISMS USED	CASEIN	PEPTONE
days			
Control		3.14	10.15
1	*A. niger*	1.12	8.76
4	*A. niger*	0	12.12
7	*A. niger*	6.20	3.94
14	*A. niger*	7.44	5.72
1	*A. ochraceus*	2.92	7.00
4	*A. ochraceus*	7.34	9.02
7	*A. ochraceus*	9.02	7.34
14	*A. ochraceus*	12.58	8.00
1	*B. mycoides*	2.92	4.68
4	*B. mycoides*	11.84	7.34
7	*B. mycoides*	12.98	16.36
14	*B. mycoides*	12.00	13.72
1	*Act. penicilloides*	5.10	12.84
4	*Act. penicilloides*	5.64	
7	*Act. penicilloides*	11.28	
14	*Act. penicilloides*	12.58	
Control	*Trypsin, 200 mgm.*	6.16	13.60
1	*Trypsin, 200 mgm.*	26.28	22.20
4	*Trypsin, 200 mgm.*	35.04	23.60

portion equivalent to 2 cc. of the original filtrate was used for the determination of amino nitrogen. The micro-method of Van Slyke was used in all cases. Frequent blanks were made in the same manner using 2 cc. of distilled water. The determinations were carefully carried out as directed by Van Slyke (1911, 1912, 1913), the amount of amino nitrogen found in the 2 cc. portions being corrected by the use of the blank, then multiplied by 50,

thus giving the total amino nitrogen present in the 100 cc. of the filtrate.

As a preliminary experiment, two quantities of Czapek's solution were made up containing 0.5 per cent casein or peptone in place of the $NaNO_3$. The solutions were sterilized and inoculated with *A. niger*, *A. ochraceus* *B. mycoides*, *Act. penicilloides*, and trypsin. The flasks were examined at the end of 1, 4, 7, 14 days for their amino nitrogen content.

The figures in table 1 reveal marked differences in the behavior of the different organisms. There is in all of the cultures, with the exception of *Act. penicilloides*, an initial decrease in the amino nitrogen content, which completely disappeared in the case of *A. niger* growing on casein, when four days old. The subsequent determinations show an increase in the amino nitrogen. This seems to confirm the work of Sears (1916), who has shown that the bacteria will first use the amino nitrogen present in the medium and only later attack the protein and split it into amino-acids.

It was thought advisable to test a larger number of organisms on peptone alone and obtain the amino nitrogen accumulation (we can not speak of amino nitrogen production in cultures where the growing organism was present, because a constant utilization of the amino nitrogen takes place in the cultures). Czapek's solution containing 2 per cent of peptone in place of the $NaNO_3$ was used. The results are presented in table 2.

On comparing the behavior of the different organisms in their power to accumulate amino nitrogen, we are at once struck by the action of *B. mycoides* and the two actinomyces used which is so distinct from that of the molds. It is quite possible that the molds were making a much more rapid growth than the bacterium or actinomyces, the amino nitrogen formed being rapidly used up by the organism or broken down to ammonia, or that the last named organisms have a greater ability to decompose the peptone into amino nitrogen compounds. On comparing the accumulation of amino nitrogen with that of ammonia, we find that there might be an indication of the fact that a great deal of the amino nitrogen is broken down to ammonia, as is seen in the

case of *A. fuscus* and *A. clavatus*, both of which showed at the end of thirteen days the lowest accumulation of amino nitrogen and the highest accumulation of ammonia nitrogen, while *B. mycoides* and *Act. violaceus-ruber* I and II gave the highest amounts of amino- and the lowest of ammonia nitrogen. This theory does not hold true for the *Act. penicilloides* which gave the highest

TABLE 2

Amino nitrogen and ammonia accumulation by microörganisms in a 2 per cent peptone solution

Milligrams of NH_2-N and NH_3-N per 100 cc. of solution

PERIOD OF INCUBATION	ORGANISMS USED	NH_2-N	NH_3-N
days			
At start		40.26	0
5	*A. niger*	24.68	7.00
13	*A. niger*	29.90	77.56
5	*A. ochraceus*	34.44	4.20
13	*A. ochraceus*	42.20	56.00
5	*A. fuscus*	28.92	5.68
13	*A. fuscus*	17.90	108.36
5	*A. clavatus*	32.19	9.80
13	*A. clavatus*	23.19	103.60
5	*Citr. glaber*	40.20	11.62
13	*Citr. glaber*	48.12	48.68
5	*P. chrysogenum*	44.36	8.12
13	*P. chrysogenum*	71.10	46.76
5	*Act. penicilloides*	72.40	6.18
13	*Act. penicilloides*	149.50	42.36
38	*Act. violaceus-ruber I*	130.63	25.20
38	*Act. violaceus-ruber II*	129.25	22.60
5	*B. mycoides*	61.70	21.00
13	*B. mycoides*	119.20	22.40

amino nitrogen and at the same time a fairly high ammonia nitrogen accumulation.

It is very possible that the organisms tested differ greatly in their power to attack the peptone and in the production of different nitrogen decomposition products; such organisms as *A. niger* and *A. fussus* do not seem to be able to allow a large accumulation of amino nitrogen, and either do not split off a great deal of it or use it up, as soon as it is formed, with the production

of ammonia as a waste product; it is characteristic that these two organisms are very rapid growers and are closely allied. At the other extreme we find organisms, such as the two strains of *Act. violaceus-ruber*, *Act. penicilloides*, and *B. mycoides*, which, without producing a very strong growth upon the medium used, showed a large accumulation of amino nitrogen containing compounds with the production of a rather small quantity of ammonia. Organisms, like *Citr. glaber* and *P. chrysogenum*, seem to allow an accumulation of amino nitrogen and ammonia, but neither in a great excess.

An experiment was started using *A. niger*, for the purpose of testing the influence of sugar upon the formation of decomposition products from proteins. Czapek's solution containing 2 per cent peptone in place of the $NaNO_3$, without the sugar, was divided into two portions: to one half 3 per cent of cane sugar was added and the second half was left without sugar, so that the peptone would have to supply to the organism both nitrogen and carbon. These solutions were distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, sterilized and inoculated with an approximately equal number of spores, then incubated at 28°C. At the end of every twenty-four hours one flask from each set was taken out from the incubator for the determination of amino and ammonia nitrogen. From the ninth day till the end of the experiment, after the ammonia had been expelled by the action of Na_2CO_3, the aeration was further continued upon the addition of NaOH. Two grams of Na_2CO_3 were added to 50 cc. of medium and aeration continued for three hours. After that period 1 to 2 grams of NaOH and 10 grams of NaCl were also added to the medium, and aeration continued further, for one hour, into a fresh quantity of standard 0.1 N H_2SO_4. The data obtained from these determinations are given in table 3. The second quantity of ammonia is either due to the decomposition of magnesium-ammonium-phosphate crystals that might have been formed in the medium and were not decomposed by the Na_2CO_3, or to the decomposition of the amide nitrogen present in the medium, as was pointed out before. Both of these may account for the large quantities of ammonia expelled by the NaOH; it might be

possible that the quantity of Na_2CO_3 or the period of aeration were not sufficient to expel all the ammonia, although the medium was shown to be strongly alkaline all through the aeration process, and a period of three hours was found to be sufficient.

TABLE 3*

The accumulation of amino nitrogen and ammonia by A. niger from 2 per cent peptone, in the presence and absence of sugar

Milligrams of NH_2-N and NH_3-N per 100 cc. of solution

PERIOD OF INCUBATION	3 PER CENT SUGAR PRESENT			SUGAR ABSENT		
	NH_3-N by Na_2CO_3	NH_3-N by NaOH	NH_2-N	NH_3-N by Na_2CO_3	NH_3-N by NaOH	NH_2-N
days						
0	0		40.60	0		40.60
1	1.00		37.74	0.84		36.01
2	1.68		31.16	1.12		32.89
3	1.68		28.60	9.10		30.03
4	2.24		27.84	11.48		32.48
5	7.00		24.94	34.72		30.16
6	12.18		25.17	49.84		33.18
7	18.62		23.45	53.90		40.04
8	28.74		22.54	70.00		32.60
9	35.60	16.90	22.27	73.62	21.14	31.98
10	48.02	17.92	19.41	76.02	25.20	30.26
11	57.24	15.26	21.13	79.94	23.80	31.68
12	71.12	14.00	20.55	87.36	23.80	36.54
13	75.60	14.00	19.18	96.04	22.40	27.98
14	78.02	16.20	17.13	100.80	23.80	29.69
15	84.00	18.20	15.98	116.06	29.40	28.55
16	89.32	19.60	18.26	129.22	30.80	21.00
17	93.28	19.60	16.54	128.94	30.60	29.50
18				129.64	38.36	21.50
19				129.92	48.02	22.34

* Certain parts of this table have been already published elsewhere (Waksman, 1917b).

The data brought out in table 3 point to a very distinct difference in the accumulation of amino and ammonia nitrogen from peptone by *A. niger* due to the presence or absence of sugar. The production of amino nitrogen is very small; there seems to be only so much of it formed as the organism needs for its development. The gradual decrease in the total amino nitrogen shows

that the peptone is broken down, a part of the decomposition products being utilized by the growing organism, while ammonia, also perhaps other nitrogenous substances, are left in the medium as waste products of the metabolism of the organism. The daily fluctuations in the content of amino nitrogen show that the splitting of the peptone may go hand in hand with the utilization of the amino nitrogen formed and its further splitting into ammonia and other products. The accumulation of the ammonia proceeds along entirely different lines than that of the amino nitrogen: while the latter gradually declines, the amount of the former gradually rises, giving at the end of 19 days as much as 50 per cent of the total nitrogen in the form of ammonia. The lack of accumulation of amino nitrogen can be explained by the fact that the organism used in this experiment grows very rapidly and probably uses all the amino nitrogen as soon as it is split off the protein molecule, or converts it into ammonia. In the case of certain other organisms, as is seen in table 2, the amino nitrogen accumulates in the medium, either due to the slow development of the organisms or to their inability to convert amino nitrogen rapidly into ammonia. The accumulation of ammonia is gradually increasing from day to day, the velocity of the reaction depending entirely on the sugar content of the medium.

Miyake (1916), using the results obtained by other investigators on bacterial activities, has shown that the processes of ammonification and nitrification are autocatalytic chemical reactions. To apply this theory to the results obtained in table 3 and also to show more clearly the difference in velocity of ammonia accumulation due to the presence or absence of sugar, the curves of autocatalysis were computed for the quantities of ammonia obtained. The tables of Dr. T. B. Robertson (1915) were used for this work, the constants having been determined from all of the observations by the method of least squares.

The curves presented in figure 1 and figure 2 are the theoretical curves obtained, while the observed quantities are given as dots. In figure 1 we can readily see that the amount of ammonia accumulated by *A. niger* from peptone in the presence of available

carbohydrates obeys the law of autocatalysis, while, in the absence of sugar, the variations are much greater, as shown in figure 2. The normal growth of the organism is disturbed in the absence of available carbohydrates when the organism has to attack the protein molecule not only for the nitrogen requirement, but also for its carbon supply. The quantities of ammonia obtained from the first to the fifth day, in the absence of sugar, are less than the calculated values, perhaps due to the fact that

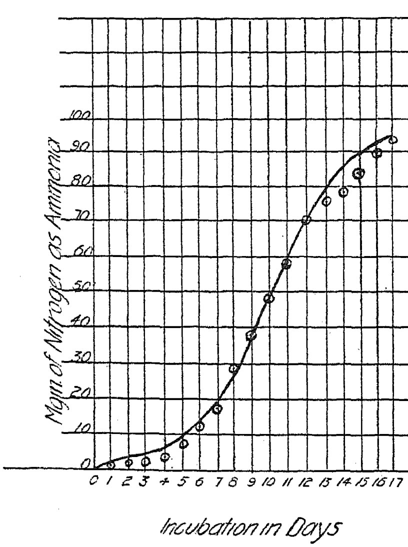

Fig. 1. Ammonia Accumulation by *A. niger* in the Presence of Sugar

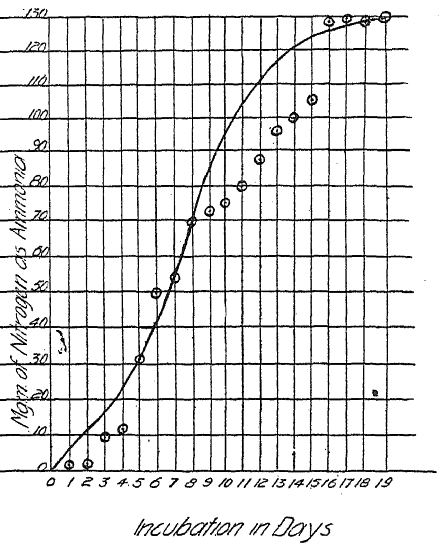

Fig. 2. Ammonia Accumulation by *A. niger* in the Absence of Sugar

it may take some time before the organism can begin to function normally when it has to derive its energy from the protein molecule; from the ninth till the sixteenth day, after the ammonia accumulation reached 70 mgm., there is another deviation of the calculated from the observed data. A similar but slighter deviation is observed also in figure 1, after the amount of ammonia reached 70 to 75 mgm. This may be either due to the exhaustion of the energy supply in the medium, or to the formation of a new autocatalytic curve. Further work on this subject continued for a much longer period of time would therefore be desirable.

As was already pointed out in another place (Waksman, 1917), the amount of available carbohydrates has a decided influence upon the metabolism of the organism and its power to accumulate ammonia from the splitting of proteins. Where sugar or other available carbohydrates are present, the organism will utilize these as a source of energy and split off from the protein only as much nitrogen as it needs for its metabolism; the quantity of ammonia accumulated in the medium will therefore be small. But, when available carbohydrates are absent, the organism will attack the protein molecule, not so much for the nitrogen as for the carbon part of it. The protein molecule decomposed into its constituent groups will lose its carbon, while only a small part of its nitrogen will be used up by the organism, because the carbon requirement of the organism is much greater than its nitrogen need, and therefore most of the nitrogen will be accumulated in the medium as a waste product, in the form of ammonia. A full discussion on this subject will be found in the article previously cited (Waksman, 1917). Doryland (1916) has shown that glucose has a detrimental effect upon ammonia production by bacteria in the decomposition of proteins; he attempted to explain this by the fact that the organisms consume some of the ammonia liberated by them from the protein, thereby leaving less ammonia in the soil or in the culture medium. Doryland assumed that the ammonia is produced by the bacteria even in the presence of glucose and is reconsumed by the organisms which are able to multiply more readily and use the more available food, although in another place Doryland himself states that "the presence of dextrose sometimes may lessen the amount of casein decomposed and amount of ammonia accumulated."

To throw more light on the production of ammonia from simpler compounds and thus perhaps indicate the possible formation of ammonia from proteins, the following experiment was started: Asparagine was added in quantities of 1, 5, 10, and 25 grams per liter of Czapek's solution, with the elimination of the $NaNO_3$. Each liter of medium was distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, and these were sterilized as usual. All the 40 flasks were inoculated with an approxi-

mately equal number of spores of *A. niger* and incubated at 28°C. At the end of twenty-four hours a flask from each set was taken out for the determination of ammonia and amino nitrogen; this was repeated at regular intervals of twenty-four hours. The data obtained are presented in table 4.

The relation between the utilization of amino nitrogen by *A. niger* and the formation of ammonia is revealed in the above table. Asparagine contains half of its nitrogen in the form of amino nitrogen, as determined by the method of Van Slyke. The

TABLE 4

The utilization of asparagine nitrogen and accumulation of ammonia by A. niger

Milligrams of NH_2-N and NH_3-N per 100 cc. of solution

PERIOD OF INCUBATION	0.1 PER CENT ASPARAGINE		0.5 PER CENT ASPARAGINE		1 PER CENT ASPARAGINE		2 5 PER CENT ASPARAGINE	
	NH_2-N	NH_3-N	NH_2-N	NH_3-N	NH_2-N	NH_3-N	NH_2-N	NH_3-N
days								
0	9.53	0	47.69	0	95.28	0	238.16	0
1	9.16	0	46.76	0	93.97	0	236.00	0
2	8.29	0.28	44.45	0.28	90.50	0.98	221.65	0.14
3	7.39	0.56	40.02	0.14	86.56	0.84	186.40	6.58
4	6.26	0	33.18	2.80	81.73	6.30	177.32	18.76
5	4.58	0	26.31	2.38	74.36	5.60	173.32	19.04
6	3.50	0	19.86	3.64	70.08	7.00	168.19	28.00
7	2.85	0.14	7.98	8.40	55.86	12.32	136.80	40.32
8	2.28	0	7.41	10.92	53.01	19.88	93.18	76.98
9	1.15	0	6.85	11.48	35.91	24.20	85.10	84.42
10	1.15	0	5.13	14.56	30.21	28.98	47.31	148.12

amount of sugar in all the media was 3 per cent. Where the amount of asparagine was small, only traces of ammonia were found and no autolysis was noticeable at the end of the experiment. The more nitrogen the medium contained, the heavier was the weight of the mycelium and the more autolysis it underwent after the optimum growth has been completed. After the organism made its optimum growth, the ammonia began to accumulate in the medium very rapidly. With the high concentration of nitrogen in the medium, the sugar became the limiting factor, and the rapid accumulation of ammonia was either due to the autolysis of the organism or to the breaking up of the

asparagine molecule for the utilization of the carbon part of it, thus leaving the nitrogen in the medium as ammonia.

Ammonia is usually looked upon as a final product in protein metabolism. That this is not the fact has recently been pointed out by Berman (1916), who stated that the determination of free ammonia as an index of protein metabolism should be condemned. As a matter of fact we have very little knowledge on that point. The accumulation of ammonia in the medium is probably due to several causes: firstly, it is an important waste product in the nitrogen metabolism of microörganisms; secondly, it is possible that, for certain organisms at least, the proteins and other nitrogenous substances must be first broken down to ammonia, this being assimilated as such by the organism, although we have no direct evidence on that point; in such a case the organism may split off more ammonia than it can use, so that a great deal of it is left in the medium. The fact that ammonia is not the final product of protein metabolism can be demonstrated in the case of those organisms that accumulate a large quantity of amino nitrogen, as was shown for *P. chrysogenum*, *Act. penicilloides*, *Act. violaceus-ruber*, and *B. mycoides*. Or it is possible that the different microörganisms behave differently in this respect, and the ammonia which may be only a waste product for some organisms may act as an intermediate product for others.

The problem of proteolytic activities of microörganisms is not such a simple one as might be thought at first. Most bacteriologists limit themselves to the study of one particular nitrogenous product, usually ammonia, and thus conclude that they are studying protein metabolism, often without taking into consideration the numerous controlling factors. In a number of experiments on ammonification in the soil by the so-called "beaker method," the author was able materially to alter the ammonia produced from a certain organic substance, using the same soil, the same moisture content, temperature and period of incubation, so as to have all the environmental conditions as much alike as possible, and merely changing one factor, such as the size of the soil particles: when the soil was placed in the beaker in small lumps, the greater aeration allowed a better development of the

fungi than of the bacteria; and the fungi, as was shown elsewhere (Waksman, 1916), produce as a group a great deal more ammonia than do the other microörganisms under the same conditions. Thus we see that by producing a slight change in the environmental conditions, we not only change the conditions for microorganic activities, but stimulate a change in the active soil flora.

The influence of any one factor is so important in controlling the amount of ammonia accumulated in the medium, that the ammonification studies, which occupy such an important place in many bacteriological investigations, particularly with soils, will have to be carefully revised. The very study of ammonification seems to be of doubtful importance, firstly due to the fact that all the controlling factors can hardly be taken into consideration in any one experiment (this refers particularly to mixtures of microörganisms); secondly, because it has not been proven as yet that ammonia is an end product in protein metabolism and under exactly what conditions it is formed as a waste product; thirdly, the ammonia produced by mixtures of microorganisms will be differently affected by different conditions which do not affect alike the different groups of microörganisms; and fourthly, if the production of ammonia by microörganisms is studied, it should not be studied by itself but in connection with the other activities of the microörganisms.

SUMMARY

1. Different organisms behave differently in their power to attack proteins and in the production of amino nitrogen and ammonia.

2. Most of the molds which grow very rapidly, as manifested by the increase in weight of their mycelium, allow a small amount of amino nitrogen to accumulate in the medium, while the amount of ammonia accumulated increases with the period of incubation.

3. Certain molds, particularly the slower growing ones, the actinomyces studied, and *B. mycoides* favor a large accumulation

of amino nitrogen in the medium and a comparatively smaller accumulation of ammonia.

4. The growth of *A. niger* upon a solution containing peptone shows that the amino nitrogen produced in the medium is used up by the organism, so that no great accumulation takes place. Ammonia, on the other hand, which seems to be a waste product of the metabolism of the organism, accumulates readily in the medium, particularly when the organism stops growing and begins to autolyze.

5. The presence of available carbohydrates checks the accumulation of ammonia in the medium, due to the fact that in their presence the organism uses only as much of the protein molecule as it needs for its nitrogen metabolism, and only a small quantity of ammonia will accumulate.

6. The process of ammonification, in the presence of available carbohydrates, is found to be an autocatalytic chemical reaction.

7. In the absence of available carbohydrates, the observed data deviated from the data calculated by the use of the curve of autocatalysis.

8. The study of ammonification is of doubtful importance in revealing to us the proteolytic activities of microörganisms, since the quantity of ammonia accumulated in the medium depends on a great number of controlling factors; it has not been proven as yet that ammonia is an end product of protein metabolism.

9. Asparagine nitrogen is rapidly converted into ammonia nitrogen, after the organism has made its maximum growth; but, where the amount of asparagine nitrogen is small, particularly in the presence of a comparatively large excess of available carbohydrates, no ammonia or only a very small quantity of it will accumulate in the medium.

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REFERENCES

Abderhalden, E., and Rona, P. 1910 Ztschr. Physiol. Chem., **46**, 179.
Berman, N. 1917 Proc. Soc. Amer. Bact., 1916 Meeting XVIII, Bact. Abstr., **1**, 60.
Brenner, W. 1914 Centrbl. Bact., II Abt., **40**, 555.
Brigham, R. O. 1917 Soil Sci., **3**, 155.
Butkewitch, W. 1903 Jahr. Wiss. Bot., **38**, 147.
Czapek, F. 1902 Beitr. Chem. Physiol. u. Pathol., **2**, 557; **3**, 47.
Deryland, C. J. T. 1916 Bull. 116, N. D. Agr. Exp. Sta.
Emmerling, O. 1902 Ber. Deut. Chem. Gesell., **135**, 2289.
Folin, O. 1902 Ztschr. Physiol. Chem., **37**, 161.
Hagem, O. 1910 Vidensk. Selsk., I Math. Naturw. Klasse, **10**, 1.
Hutchinson, H. B., and Miller, N. H. J. 1912 Jour. Agr. Sci., **4**, 282.
Itano, A. 1916 Bull. 167, Mass. Agr. Exp. Sta.
Miyake, K. 1916 Soil Sci., **2**, 481.
Potter, R. S., and Snyder, R. S. 1915 Research Bull. 17, Iowa Agr. Exp. Sta.
Rettger, L. F., Berman, N., and Sturges, W. S. 1916 Jour. Bact., **1**, 15.
Robertson, T. B. 1915 Univ. California Pub., Physiology, **4**, 211.
Schreiner, O., and Shorey, E. C. 1910 Jour. Biol. Chem., **8**, 381.
Sears, H. J. 1916 Jour. Inf. Dis., **19**, 105.
Skinner, J. J. 1912 a Intern. Cong. App. Chem. VIII, Science, **15**, 253.
Skinner, J. J. 1912 b Bot. Gaz., **54**, 152.
Steel, M. 1910 Jour. Biol. Chem., **8**, 365.
Van Slyke, D. D. 1911 Jour. Biol. Chem., **9**, 185.
Van Slyke, D. D. 1913 Jour. Biol. Chem., **16**, 121.
Waksman, S. A. 1916 Soil Sci., **2**, 103.
Waksman, S. A. 1917 a Soil Sci., **3**, 565.
Waksman, S. A. 1917 b Jour. Amer. Chem. Soc., **39**, 1503.

A MODIFICATION OF THE TECHNIQUE OF THE VOGES AND PROSKAUER REACTION

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Summary. The use of Syracuse watch glasses, 1 cc. of glucose potassium phosphate broth—incubated for forty-eight hours at 30°C.—and 0.5 cc. of a 45 per cent solution of sodium hydroxide, permits the development of a definite color reaction in this test in a maximum period of one and one-half hours after the addition of the latter solution.

While engaged in a study of the members of the colon group in the local water supplies a comparison was made between the methods for this test as described in Clemesha's "The Bacteriology of Surface Waters in the Tropics" and in the latest edition of "Standard Methods." Clemesha employed a medium consisting of 10 grams of peptone and 5 grams of glucose in 1000 cc. of water with the following procedure:

> The Voges and Proskauer reaction is completed by adding a few drops of a very concentrated solution of caustic potash after forty-eight hours' (or more) incubation. The red colouration nearly always appears within an hour, but in some cases it is delayed. The tubes are, however, kept for twenty-four hours after the caustic potash has been added.

On a following page of his book he wrote:

> Some care has to be exercised, as in all similar reactions, for in some cases the colour is rather faint. If, however, a very small quantity (2 or 3 drops) of a saturated solution of caustic potash is used, instead

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of a large quantity of dilute solution, the amount of fluid in the tube is not increased, and the colour very seldom fails to appear in sufficient quantities to be easily recognizable in two hours.

In the 1917 edition of "Standard Methods of Water Analysis," a medium consisting of 5 grams of Witte's peptone, 5 grams of c.p. glucose and 5 grams of dipotassium phosphate (K_2HPO_4) in 100 cc. of water, is recommended for the methyl red and Voges and Proskauer tests with the following procedure, after incubation at 30°C. for five days and after the former test has been made: "To the remaining 5 cc. of medium add 5 cc. of a 10 per cent solution of potassium hydroxide. Allow to stand over night. A positive test is indicated by an eosin pink color."

In the case of both media, after the addition of the sodium hydroxide, the tubes removed from the incubator in the afternoon were returned to it or allowed to stand in the laboratory until the morning of the following day. After a few parallel tests had been made it was observed that, with the same cultures, positive Voges and Proskauer reactions did not always develop in the two media. As a result of this disagreement a study was made of the factors which were thought to exert the greatest influence on the reaction, namely, the concentration of the sodium hydroxide solution and the optimum interval of time necessary for the development of the pink color.

Without giving the details of all the experiments it was found that the addition of 0.5 cc. of a 45 per cent solution (1 pound per liter of water) of sodium hydroxide to about 5 cc. of the media gave sharper and more distinct colors than that of 5 cc. of a 10 per cent solution. At the same time it was observed, in many of the tubes in which positive reactions developed at the end of five hours, that the pink color disappeared on standing over night; that the intensity of the pink color varied with different cultures; that the permanency of the color was usually proportional to the intensity; and that the color in the majority of cases developed in an interval of two hours after the addition of sodium hydroxide. The following table furnishes a comparison of the positive Voges and Proskauer reactions resulting from the use

of the two media with 283 strains of lactose fermenting organisms which were negative to methyl red. The development of the pink color was noted at the end of 1, 2, 5, and 20 to 25 (over night) hours after the addition of 0.5 cc. of a 45 per cent solution of sodium hydroxide to the tubes of Clemesha's medium and 5 cc. of a 10 per cent solution to the tubes of glucose potassium phosphate medium. The positive reactions are expressed in percentages of the 283 methyl red negative organisms.

From this table it is seen that the correlation percentages are much higher in the case of Clemesha's medium; that the five hour period of reaction with the sodium hydroxide is the optimum interval of time for the development of the pink color;

TABLE 1

Percentages of positive Voges and Proskauer reactions with 283 methyl red negative reactions

	TUBES			
	Hours after addition of sodium hydroxide			
	1	2	5	20–25
Glucose potassium phosphate broth, five days at 30°C	31.1	43.5	56.0	36.0
Clemesha's medium, two days at 37.5°C	66.3	70.2	79.9	70.6

and that the over night period gives erroneous results on account of the fading of the pink color. All tubes in which a noticeable pink color developed were called positive. Part of the negative tubes were colorless and part were characterized by a yellowish green color.

From the work of Harden and Walpole it appeared that oxidation plays an important part in the development of the pink color. In order to hasten the oxidation the more intimate exposure of a portion of the incubated culture appeared to be the next step in the investigation. Accordingly Syracuse watch glasses measuring 5 cm. in diameter by 8 mm. in depth were employed as containers. In such a dish 1 cc. of liquid is spread over an area of about 20 sq. cm. with a resulting depth of liquid of about

0.05 cm. After several trials a combination of 1 cc. of the culture and 0.5 cc. of a 45 per cent solution of sodium hydroxide was found to produce a deep pink color in about one hour's interval after the addition of the latter. The maximum intensity of the color developed between the first and second hour so that the readings were taken at both intervals. After the latter interval the color started to fade. The use of the Syracuse watch glasses was started in April, 1917.

Table 2 gives a comparison of the Voges and Proskauer reactions developing from the same cultures in glucose potassium phosphate broth incubated at 30°C. for five days and at 37.5°C. for two days and in Clemesha's medium incubated for two days

TABLE 2

Percentages of 262 methyl red negative cultures giving positive Voges and Proskauer reactions

	Dishes		Tubes			
	Hours after addition of sodium hydroxide					
	1	2	1	2	5	20–25
Glucose potassium phosphate broth:						
Five days at 30°C.	76	73	31	49	58	37
Two days at 37.5°C.	82	85	45	62	67	65
Clemesha's medium 2 days at 37.5°C.	88	89	65	73	83	74

at 37.5°C. The lactose fermenters employed in these tests were derived from Tapir feces, grass, and water samples.

From this table it is evident that:

1. Greater percentages of positive Voges and Proskauer reactions were obtained in the Syracuse watch glasses (columns headed "Dishes") than in the test tubes.

2. Some of the Voges and Proskauer reactions, positive in the tubes at the end of five hours, faded on standing an additional fifteen to twenty hours.

3. In the case of Clemesha's medium the percentages of positive Voges and Proskauer reactions were 89 and 83, respectively, in the Syracuse watch glasses at the end of two hours and in the test tubes at the end of five hours. With the glucose potassium phosphate medium, at both periods of incubation, the percent-

ages of positive Voges and Proskauer reactions in the two containers showed a marked disagreement.

4. Based on the number of positive Voges and Proskauer reactions, Clemesha's medium ranks first, the glucose potassium phosphate medium second when inoculated at 37.5°C. for forty-eight hours, and third when inoculated at 30°C. for five days.

In the course of this study it became evident that a considerable number of organisms would give positive Voges and Proskauer reactions with Clemesha's medium and negative results with the glucose potassium phosphate medium. Furthermore the latter, when inoculated with the same culture, did not always yield positive Voges and Proskauer reactions after incubation at 30°C. for five days and 37.5°C. for two days. In seeking an explanation for this non-agreement the influences of temperature and incubation period were studied by inoculating large quantities of glucose potassium phosphate broth and Clemesha's medium with various cultures so that portions could be removed for testing the Voges and Proskauer reactions at twenty-four hour intervals for a period of five days. It was found that: forty-eight hours' incubation was sufficient time for the development of acetyl-methyl-carbinol and a longer incubation period exerted an unfavorable influence. In general, the intensities of the colors which developed after the addition of sodium hydroxide diminished as the incubation period increased, faint and very faint pink colors appearing and shading, in some cases, to brown or yellow. At the end of forty-eight hours colors of a uniform strong pink shade appeared and doubtful colors were absent. A considerable percentage of cultures yielded positive Voges and Proskauer reactions at the end of twenty-four and forty-eight hours and negative at the end of five days. Table 3 contains the results of some tests made to illustrate the influence of the period of incubation on the development of positive Voges and Proskauer reactions. From each 100 cultures tested by the Voges and Proskauer reaction the tests indicated were obtained in the respective media at the various incubation periods.

Table 2 shows that cultures in Clemesha's medium incubated at 37.5°C. for two days yield a larger percentage of positive Voges and Proskauer reactions (see columns headed "Dish"—

two hours) than those inoculated into glucose potassium phosphate and incubated at 30°C. for five days and 37.5°C. for two days, the respective percentages being 89, 73 and 85. The data included in table 3 and other tests not included in this article show that glucose potassium phosphate broth incubated at 30°C. for forty-eight hours is superior to the same medium, and also to Clemesha's medium, when incubated two days at 37.5°C. for yielding positive Voges and Proskauer reactions.

TABLE 3

MEDIA AND TEMPERATURES	INCUBATION PERIOD IN DAYS				
	1	2	3	4	5
Glucose potassium phosphate broth:					
30°C.	82	82	82	78	67
37.5°C.	78	75	58	36	21
Clemesha's medium 37.5°C.	64	72	70		

Bacto-peptone furnished by the Digestive Ferments Company has been used entirely in the media mentioned in this article. Due to delay in obtaining other brands of peptone a comparative study could not be made in time to be included in this paper but the results will be presented later in connection with other factors influencing this reaction.

CONCLUSIONS

The use of 0.5 cc. of a 45 per cent solution of sodium hydroxide, Syracuse watch glasses, and 1 cc. of the culture results in the development of a greater number of positive Voges and Proskauer reactions in a shorter interval of time than the method given in the latest (1917) edition of "Standard Methods of Water Analysis."

An incubation period of two days at 30°C. is recommended in place of that of five days in order to obtain the maximum number of positive Voges and Proskauser reactions from glucose potassium phosphate broth. In order to avoid separate cultures for this test 1 cc. portions may be withdrawn from those incubated for the methyl red test.

OZENA AND DISTEMPER: COMPARATIVE STUDY OF COCCOBACILLUS FOETIDUS-OZAENAE AND BACILLUS BRONCHISEPTICUS

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The question of the relationship of ozena to distemper has been frequently discussed, both in print and otherwise. Many observers consider the dog a carrier of an organism common to both diseases; and the patients themselves are often of the same opinion as they will frequently volunteer information bearing on their association with dogs, special emphasis being laid on a coincident infection of the animal with distemper sometime in the early part of the disease.

Perez (1913) carried out some experimental work along this line and suggested that the infection in ozena probably originates from dogs, and, as a result of his work, cultures from these animals were included in an ozena vaccine upon which Hofer and Kofler (1914), collaborators of Perez, were experimenting.

Horn and Victors (1916) in this country, considering *Coccobacillus foetidus-ozaenae* (Perez) as the principal etiological factor in ozena and *Bacillus bronchisepticus* (Ferry) as the cause of distemper, have even attempted to prove a relationship between the above mentioned organisms and claim to have found a positive complement fixation between them. The question, however was left in rather an unsettled condition as may be seen by the following statement made by them:

"*Bacillus bronchisepticus*, exhaustively studied by Ferry, M'Gowan, Torrey and Rahe, and determined by them as being the specific organism of canine distemper, morphologically almost identical and biologically in many instances similar to the Perez organism, and the

advanced hypothesis that the infection of fetid ozena is carried by dogs, presents an interesting complex."

It is not the province of this paper to discuss nor to attempt to offer any proof pro or con as to the etiology of distemper in the dog or of ozena in the human subject (that phase of the question having been taken up by the several authors elsewhere); but rather to attempt to answer the question, as to the relationship of Perez' organism to *B. bronchisepticus*, which has been so significantly brought forward by Horn and Victors.

If Perez' bacillus is the cause of ozena and it is demonstrated to be the same as, or closely related to, *B. bronchisepticus*, an extremely important point has been established and its bearing on the prophylaxis and management of ozena can hardly be estimated at the present time, since distemper is most widely distributed among dogs and other small animals, especially laboratory animals, such as the rabbit and guinea pig. Accordingly, the authors, using several typical strains of both organisms, have carried on a large number of comparative morphological, cultural and serological tests in an attempt to determine, if possible, the characteristics common or perhaps, to be more exact, not common to both organisms.

For the morphological and cultural characteristics of *B. bronchisepticus* those originally published by one of us (Ferry, 1910, 1911) and corroborated by M'Gowan (1911) and Torrey and Rahe (1913) are taken as characteristic; and for *Coccobacillus foetidus-ozaenae* only those described by Ward (1917) are considered, as they apply directly to the strains under discussion in this work. Unfortunately, there does not seem to be a unanimity of opinion among writers relative to all reactions of this organism. The question of motility seems to be still undecided. The four strains under discussion, however, were found by the authors to be motile, although not so progressively active as *B. bronchisepticus*. In this instance the authors do not agree with Ward, who claims that the European strains are non-motile.

SUMMARY OF CULTURAL REACTIONS

1. *B. ozaenae* may be readily differentiated from *B. bronchisepticus* by any one of the following reactions; by growth on potato, on Loeffler's blood serum and in litmus milk, by the fermentation reaction in glucose media and by the indol reaction in Dunham's solution.

2. Less important differentiating characteristics are found in morphology, motility, colony formation and odor.

Differentiating cultural characteristics

	PEREZ' BACILLUS	B. BRONCHISEPTICUS
Morphology	Small coccoid bacillus, no filaments	Small narrow bipolar bacillus. Filaments in liquid media
Motility	Sluggish	Active
Colony	Old colony, lobate	Round, entire
Loeffler's blood serum	No proteolysis, cream to greenish yellow	No proteolysis, old cultures tan color
Potato	Limited, faintly yellow	Spreading, tan
Plain broth	Uniform turbidity, characteristic nauseating odor	Uniform turbidity, odor of stale bread
Litmus milk	Small amount of acid	Decided alkalinity
Dunham's	Indol positive	Indol negative
Fermentation Glucose	Acid—gas	Alkaline—no gas

AGGLUTINATION REACTIONS WITH B. BRONCHISEPTICUS AND PEREZ' BACILLUS

Rabbits have been treated with vaccines of the following cultures of *B. bronchisepticus* and Perez' Bacillus and the sera obtained for the purpose of making cross agglutination tests between these two organisms.

B. bronchisepticus no. 36, from dog *B. bronchisepticus* no. 123, from monkey *B. bronchisepticus* from human	}	Isolated by N. S. F.
Perez' Bacillus no. 1		Isolated by Ward
Perez' Bacillus no. 2, "Hofer" strain Perez' Bacillus no. 3, "Vienna" strain	}	European strains
Perez' Bacillus no. 4		Isolated by Ward

Vaccines. Each organism was transplanted daily for several days on plain agar; then twenty-four hour growths were washed off in 0.85 per cent salt solution plus 0.2 per cent trikresol (5 cc. per culture). Each vaccine was thoroughly shaken in a mechanical shaker and two days later tested for sterility.

Production of antisera. Before the rabbits were injected each was bled from an artery in the ear and the serum tested for agglutinins against both *B. bronchisepticus* and Perez' Bacillus. No rabbit showed an agglutination titre of above 1 in 20 against either organism.

TABLE 1

DILUTIONS	SERUM FROM RABBIT 32, TREATED WITH B. BRONCHISEPTICUS NO. 36 (DOG), AGAINST SUSPENSIONS OF:						
	B. bronchisepticus			Perez' Bacillus			
	No. 36 (dog)	No. 123 (monkey)	Human	No. 1	No. 2	No. 3	No. 4
1–10	+++	+++	+++	–	–	–	–
1–20	+++	+++	+++	–	–	–	–
1–40	+++	+++	+++	–	–	–	–
1–80	+++	+++	+++	–	–	–	–
1–200	+++	+++	+++	–	–	–	–
1–400	+++	+++	+++	–	–	–	–
1–800	+++	+++	+++	–	–	–	–
1–1600	+++	+++	+++	–	–	–	–
1–2000	+++	+++	+++	–	–	–	–
1–3200	+++	+++	+++	–	–	–	–
1–6400	+	+	+	–	–	–	–
1–10000	–	–	–	–	–	–	–
1–20000	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–

+++ = Complete agglutination with all organisms clumped and fluid clear.
++ = Partial agglutination, with marked clumping but fluid not cleared up.
+ = Slight agglutination but still with positive clumping.
– = No clumping and no clearing.

Each rabbit received three intravenous injections of 0.5, 1.0 and 2.0 cc. of killed vaccine three days apart, and was bled to death on the fourth day after the last dose. To the sera was added 0.2 per cent trikresol.

Preparation of suspensions for agglutination tests. Each organism was transplanted daily, on plain agar, for ten days; twenty-four hour growths planted on plain agar in whiskey flasks; incubated eighteen hours; growths washed off in physiologic salt solution plus 0.5 per cent formalin. The suspensions were shaken for two hours; two days later tested for sterility; then filtered three times through filter paper. Each was later diluted with salt solution plus 0.5 per cent formalin to correspond in density to our standard suspension of *B. bronchisepticus*,

TABLE 2

DILUTIONS	SERUM FROM RABBIT 36, TREATED WITH PEREZ' BACILLUS NO. 1. AGAINST SUSPENSIONS OF:						
	Perez' Bacillus				*B. bronchisepticus*		
	No. 1	No. 2	No. 3	No. 4	No. 36 (dog)	No. 123 (monkey)	Human
1-10	++	+++	++	++	+	+	+
1-20	+++	+++	+++	+++	–	–	–
1-40	+++	+++	+++	+++	–	–	–
1-80	+++	+++	+++	+++	–	–	–
1-200	+++	+++	+++	+++	–	–	–
1-400	+++	+++	+++	+++	–	–	–
1-800	+++	+++	+++	+++	–	–	–
1-1600	+++	+++	+++	+++	–	–	–
1-2000	+++	+++	+++	+++	–	–	–
1-3200	+++	++	+++	+++	–	–	–
1-6400	+	+	–	++	–	–	–
1-10000	–	–	–	–	–	–	–
1-20000	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–

which contains about 2,000,000,000 organisms per cubic centimeter. Perfectly homogenous suspensions of all the strains used were produced by this method.

Agglutination tests. The serum was diluted with physiologic salt solution and each tube contained 0.5 cc. suspension plus 0.5 cc. diluted serum. The tests were incubated at 37°C. and readings made at the end of twenty-four hours.

Tables 1, 2 and 3 show the results of the agglutination experiments.

TABLE 3

CROSS AGGLUTINATION TESTS WITH B. BRONCHISEPTICUS AND PEREZ' BACILLUS. SUSPENSIONS

ANTISERA	*B. bronchisepticus* No. 36 (dog)	*B. bronchisepticus* No. 123 (monkey)	*B. bronchisepticus* Human	Perez' Bacillus No. 1	Perez' Bacillus No. 2	Perez' Bacillus No. 3	Perez' Bacillus No. 4
B. bronchisepticus							
No. 36 Rabbit 32	**1–6400**			–	–	–	–
No. 36 Rabbit 2	**1–10000**	1–20000	1–10000	–	–	–	–
No. 123 Rabbit 33		**1–6400**		–	–	–	–
No. 123 Rabbit 34		**1–6400**		–	–	–	–
No. 123 Rabbit 4	1–10000	**1–10000**	1–20000				
Human Rabbit 5			**1–6400**	–	–	–	–
Human Rabbit 6	1–10000	1–10000	**1–10000**	–	–	–	–
Perez' Bacillus							
No. 1 Rabbit 36	1–10	1–10	1–10	**1–6400**	1–6400	1–3200	1–6400
No. 1 Rabbit 37	–	–	–	**1–6400**			
No. 2 Rabbit 38	–	1–10	–	1–10000	**1–6400**	1–6400	1–10000
No. 2 Rabbit 39	–	–	–		**1–3200**		
No. 3 Rabbit 40	–	–	–	1–10000	1–6400	**1–10000**	1–10000
No. 3 Rabbit 41	1–10	–	–			**1–6400**	
No. 4 Rabbit 42	1–10	1–20	1–10	1–6400	1–6400	1–6400	**1–10000**
No. 4 Rabbit 43	1–20	1–20	1–20				**1–10000**

SUMMARY OF AGGLUTINATION EXPERIMENTS

1. Sera of high agglutination titre (1–3200 to 1–10000) were produced in rabbits by three intravenous injections of killed unheated vaccines of *B. bronchisepticus* and of Perez' Bacillus

2. All the strains of Perez' Bacillus used were identical by agglutination test—each antiserum agglutinating all strains equally well.

3. All strains of *B. bronchisepticus* were identical.

4. There was no cross agglutination between *B. bronchisepticus* and Perez' Bacillus.

COMPLEMENT FIXATION REACTIONS WITH B. BRONCHISEPTICUS AND PEREZ' BACILLUS

Complement fixation tests were made with the same antisera as used for the agglutination reactions.

Technic. The volume of all the complement fixation tests and all titrations was 4.5 cc. The method in general was that given by Kolmer in "Infection, Immunity and Specific Therapy."

For the hemolytic system, *sheep corpuscles in 5 per cent solution* (the blood was defibrinated with beads, measured, washed five times in salt solution, diluted back to original volume and 5 cc. of this used in 100 cc. salt solution), *rabbit amboceptor*, and *guinea-pig complement* in a 1 in 20 dilution (serum from at least two pigs being mixed) were used. The amboceptor was titrated daily with each complement and corpuscle suspension and that amount which showed just complete hemolysis in one hour at 37°C. was taken as the unit. In the tests one and a half times the unit was used.

Each antigen was titrated for its anticomplementary and its antigenic unit. In the tests, one-half to one-fourth the anticomplementary unit was used.

Each antiserum was tested to determine the smallest amount which, with its homologous antigen, completely inhibited hemolysis.

The test. Antigen plus serum plus complement was incubated one hour at 37°C. then amboceptor and cells were added and incubated from one to one and one-half hour depending on the antigen controls. The tests were then placed in the ice-chest and read the following morning.

Antigens. *B. bronchisepticus* and Perez' Bacillus were transplanted daily for several days, then planted on plain agar in whiskey flasks; incubated for twenty-four hours at 37°C.; the growths washed off in sterile distilled water (about 50 cc. per flask); shaken in a mechanical shaker for forty-eight hours; brought up in a water bath to 56°C. and incubated at that temperature over night; the antigens were then filtered through asbestos and to nine parts of clear filtrate one part of 8.5 per

cent salt solution plus 5 per cent formalin was added. The antigens were then kept in the ice chest.

Cross titrations. Cross titrations were made between the several strains of *B. bronchisepticus*, the several strains of Perez' Bacillus and between *B. bronchisepticus* and Perez' Bacillus.

Each serum was tested against each of the seven antigens and on the same day, all the tests being run parallel.

Tables 4, 5 and 6 show the results of the complement fixation tests.

TABLE 4

AMOUNT OF SERUM	SERUM FROM RABBIT 32, TREATED WITH B. BRONCHISEPTICUS NO. 36 (DOG). AGAINST ANTIGENS OF:						
	B. bronchisepticus			Perez' Bacillus			
	No. 36 (dog)	No. 123 (monkey)	Human	No 1	No. 2	No. 3	No. 4
cc.							
0.1	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.	C. H.
0.05	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.	C. H.
0.01	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.	C. H.
0.008	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.	C. H.
0.005	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.	C. H.
0.004	C. I.	Marked I	Marked I	C. H.	C. H.	C. H.	C. H.
0.003	C. I.	Marked I	Marked I	C. H.	C. H.	C. H.	C. H.
0.002	C. I.	Slight I	Slight I	C. H.	C. H.	C. H.	C. H.
0.001	Slight I.	C. H.	C. H.	C. H.	C. H.	C. H.	C. H.
Controls:							
Antigen...........	C. H.	C. H.	C. H.	C. H.	C. H.	C. H.	C. H.
Serum.............	C. H.						
Hemolytic.........	C. H.						
Corpuscle.........	No. H.						

C. H. = Complete hemolysis.
C. I. = Complete inhibition of hemolysis.

SUMMARY OF COMPLEMENT FIXATION TESTS

1. Sera of high complement-fixing titre were produced in rabbits by three intravenous injections of killed unheated suspensions of *B. bronchisepticus* and Perez' Bacillus.

2. The four strains of Perez' Bacillus gave identical complement fixation reactions.

TABLE 5

AMOUNT OF SERUM	SERUM FROM RABBIT 36, TREATED WITH PEREZ' BACILLUS NO. 1. AGAINST ANTIGENS OF:						
	Perez' Bacillus				*B. bronchisepticus*		
	No. 1	No. 2	No. 3	No. 4	No. 36 (dog)	No. 123 (monkey)	Human
cc.							
0.01	C. I.	C. I.	C. I.	C. I.	C. H.	C. H.	C. H
0.05	C. I.	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.
0.01	C. I.	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.
0.008	C. I.	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.
0.005	C. I.	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.
0.004	C. I.	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.
0.003	C. I.	C. I.	C. I.	C. I.	C. H.	C. H.	C. H.
0.002	Marked I.	Marked I.	Marked I.	Marked I.	C. H.	C. H.	C. H.
0.001	Slight I.	C. H.	Slight I.	C. H.	C. H.	C. H.	C. H.
Controls:							
Antigen.......	C. H.	C. H.	C. H.	C. H.	C. H.	C. H.	C. H
Serum.........	C. H.						
Hemolytic.....	C. H.						
Corpuscle.....	No H.						

TABLE 6

Cross complement fixation tests with B. bronchisepticus and Perez' Bacillus

ANTISERA	ANTIGENS						
	B. bronchisepticus			Perez' Bacillus			
	No. 36 (dog)	No. 123 (monkey)	Human	No. 1	No. 2	No. 3	No. 4
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
B. bronchisepticus							
No. 36 Rabbit 32..............	**0.002**	0.005	0.005	—	—	—	—
No. 36 Rabbit 2..............	**0.004**	0.008	0.008	—	—	—	—
No. 123 Rabbit 33..............	0.008	**0.003**	0.003	—	—	—	—
No. 123 Rabbit 34..............	0.05	**0.008**	0.008	—	—	—	—
Human							
Human Rabbit 5..............	0.05	0.003	**0.003**	—	—	—	—
Human Rabbit 6..............	0.05	0.005	**0.005**	—	—	—	—
Perez' Bacillus							
No. 1, Rabbit 36................	—	—	—	**0.003**	0.003	0.003	0.003
No. 2, Rabbit 38................	—	—	—	0.003	**0.003**	0.003	0.003
No. 3, Rabbit 40................	—	—	—	0.003	0.003	**0.003**	0.003
No. 4. Rabbit 42................	—	—	—	0.002	0.002	0.002	**0.002**

3. The human and monkey strains of *B. bronchisepticus* gave identical complement fixation reactions, but these two strains cross-fixed with the dog strain only with larger amounts of serum.

4. Immune sera of *B. bronchisepticus* did not cross-fix with antigens of Perez' Bacillus.

5. Immune sera of Perez' Bacillus did not cross-fix with antigens of *B. bronchisepticus*.

CONCLUSIONS

According to the cultural reactions and the agglutination and complement fixation tests there appear to be no reasons why Perez' Bacillus should be confused with *B. bronchisepticus*. The organisms can be differentiated from each other by any one or more of the above methods.

The results of Horn and Victors with the complement fixation test have not been corroborated by us.

REFERENCES

FERRY, N. S. 1910 A preliminary report of the bacterial findings in canine distemper. Am. Vet. Rev., **37**, 499.

FERRY, N. S. 1911 Etiology of canine distemper. J. Infect. Dis., **4**, 399.

HOFER, G., AND KOFLER, K. 1914 Weitere Mitteilungen über die Ergebnisse der Vakzinationstherapie bei genuiner Ozäna mit einer aus dem Coccobacillus fötidus ozänaee Perez. Arch. f. Laryngol., **29**, 1.

HORN, H., AND VICTORS, E. A. 1916 A contribution to the bacteriology of the so-called Coccobacillus fetidus ozenae (Perez)—, with additional notes on the treatment of clinical ozena by means of polyvalent vaccines made from the same organisms. Ann. Otol., Rhinol. Laryngol., **25**, 251.

M'GOWAN, J. P. 1911 Some observations on a laboratory epidemic, principally among dogs and cats, in which the animals affected presented the symptoms of the disease called "distemper." J. Path. & Bacteriol., **15**, 372.

PEREZ 1913 Die Ozäna eine infectiöse und kontagiöse Krankheit. Berl. klin. Wchnschr., **52**, 2411.

TORREY, J. C., AND RAHE, A. H. 1913 Studies in canine distemper. J. Med. Research, **27**,291.

WARD, H. C. 1917 Coccobacillus (foetidus) ozaenae of Perez. J. Infect. Dis. **21**, 338.

STUDIES ON THE PROTEOLYTIC ENZYMES OF SOIL FUNGI AND ACTINOMYCETES[1]

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The proteolytic enzymes of microörganisms have been studied by a number of investigators; but most of the data obtained are merely qualitative in nature. A full bibliography on the investigations concerning bacterial enzymes can be found in the work of Fuhrman (1907); the investigations of the enzymes of molds have been reviewed by Wehmer (1907) and by Dox (1910). Mention will here be made only of those investigations which have a direct bearing upon the work at hand.

ORGANISMS USED IN THE PRESENT STUDY

A number of organisms isolated from different soils were used for this work, although the greatest amount of work was devoted to the first named organism.

Aspergillus niger van Tieghem. This organism was isolated from a sandy loam soil on the College Farm, at New Brunswick, New Jersey. Methods of isolation and the description of soils, for this as well as for the following organisms, can be found in another place (Waksman, 1916). This organism was selected for the work because many of the previous investigations on enzymes of molds have been conducted with it. Since its identification is not difficult, we may suppose that previous investigators used the organism originally described by van Tieghem, although, as was recently shown by Thom (1916), the organism

[1] The data presented in this paper form Part II of the dissertation presented by the author for the degree of Doctor of Philosophy, University of California, December, 1917.

belongs to the group of black Aspergilli which have not been well differentiated before.

Aspergillus ochraceus Wilhelm. A preliminary description of this organism has appeared in another place (Waksman, 1916), as C. 19. The organism revealed peculiar metabolic processes and a number of investigations were conducted with it.

Aspergillus fuscus Schiemann.

Aspergillus clavatus Desmazières.

Citromyces glaber Wehmer.

Penicillium chrysogenum Thom.

Actinomyces griseus (Krainsky) Waksman and Curtis.

Actinomyces violaceus-ruber Waksman and Curtis.

Actinomyces sp. 101 (*virido-chromogenus?*) Krainsky Waksman and Curtis. A full description of this organism will soon appear.

Actinomyces californicus Waksman and Curtis.

METHODS USED IN THE INVESTIGATION

The organisms were grown on Czapek's solution composed as follows:

$NaNO_3$	2.0 grams
K_2HPO_4	1.0 gram
KCl	0.5 gram
$MgSO_4$	0.5 gram
$FeSO_4$	0.01 gram
Cane sugar	30.0 grams
Distilled water	1000 cc.

In addition another medium was made up by substituting 20 grams peptone for the 2 grams $NaNO_3$ per liter. This medium is called Peptone-Czapek solution. The media were distributed in 100 cc. portions in 200 cc. Erlenmeyer flasks, plugged and sterilized for fifteen minutes at 15 pounds pressure. The flasks were then inoculated and incubated at 28°C. At the end of the proper incubation period, the cultures were filtered through No. 584 folded Schleicher & Schüll filters. The filtrate was used for the study of the exoenzymes; while the mycelium was washed with distilled water, and then treated by the acetone method, as used by Dox (1910), Scales (1914), and others. Twenty

cubic centimeters of the liquid or a portion of the treated mycelium, equivalent to 0.5 to 0.8 gram of the dried substance per 100 cc. of 1 per cent solution of substratum were used. In several cases Grübler's trypsin and pepsin were used for comparison of the enzyme activities. The protein solutions were heated to boiling so as to kill the proteolytic enzymes that they might possibly contain. In all cases a check was made by boiling the protein with the added enzyme, then incubating it in parallel with the enzyme cultures. The proper reaction was always obtained by the use of 0.1 N KOH or HCl. Both chloroform and toluene were used to keep the solutions sterile. The substrata, plus enzyme, as well as the blanks, were incubated at 37°C. After the proper incubation period, 2 cc. portions of the solution were used for the determination of amino nitrogen by the use of the micro-apparatus of Van Slyke (1911, 1913). Duplicate determinations were made, but these always checked very well, so that only the averages are given in the tables. The amino nitrogen content was always figured back to 100 cc. of solution.

As substrata the following proteins were used: (1) Merck's peptone; (2) casein prepared after Hammarsten, each gram at first being dissolved in 8 cc. of 0.1N KOH, then brought to the proper reaction by the use of 0.1N HCl; (3) crystalline egg-albumen prepared from fresh eggs using the method of Hopkins and Pincus (1898); (4) Merck's fibrin, 1 gram per 100 cc. of water. The concentration of all the substrata was made up in such a manner as to constitute 1 per cent solutions upon the addition of the proper amount of enzyme.

It appeared desirable to obtain, first, an insight into the nature of the exo- and endoenzymes of several microörganisms. Dox (1910) claimed that the same enzyme may, in the earlier stages of growth of the organism, function within the cell, and later be liberated into the substratum as an extracellular enzyme. Abderhalden and Pringsheim (1910) maintained that the absence of enzymes in the liquid obtained by the Buchner method cannot serve as a criterion for the absence of particular enzymes; the mycelium itself from the culture has to be studied for the presence of the enzyme, which may not have diffused into the liquid medium.

A number of organisms were grown on Czapek's solution, for a period of eighteen to sixty days, the slower growing organisms, such as the actinomycetes, requiring a longer incubation period. At the end of that period, the cultures were filtered and the mycelium treated by the acetone method. This method consists in washing the mycelium a few times with distilled water, then drying it between filter paper, cutting it into fine pieces, and covering it for ten minutes with acetone; the mycelium is then dried again and covered for three minutes with ether, and finally dried over sulfuric acid at 35 to 40°C. One per cent peptone and casein solutions, neutral to litmus, were used as substrata. Fifty milligrams of treated actinomyces mycelium and 800 mgm. of fungus mycelium were taken for the inoculation of each flask for the study of the endoenzymes. Determinations of the amino nitrogen formed as a result of the action of the enzyme upon the substratum were made at the end of four days. The blank was subtracted from each determination.

TABLE 1

Action of exo- and endoenzymes of microörganisms upon casein and peptone

ORGANISMS USED	AGE OF CULTURE	MILLIGRAMS OF NH_2=N PER 100 CC. OF SOLUTION			
		1 per cent peptone		1 per cent casein	
		Exoenzyme	Endoenzyme	Exoenzyme	Endoenzyme
	days				
Control		21.36	20.26	7.26	6.20
A. niger	18	22.11	41.32	10.48	29.10
A. ochraceus	18	26.19	47.72	19.20	37.83
Citr. glaber	18	24.44	47.14	17.46	55.29
P. chrysogenum	8	50.40		16.90	
P. chrysogenum	60	59.36	62.27	70.42	68.09
Act. griseus	60	37.25	27.35	52.38	34.92
Act. sp. 101	60	50.63	37.25	52.38	75.66
Act. violaceus-ruber	60	20.95	38.76	55.29	43.65
Trypsin: 200 mgm.		47.12		62.12	

It is seen from table I that both the exo- and endoenzymes of the microörganisms studied, when these organisms have been grown on a non-protein medium, can split casein and peptone

to amino-acids or to compounds containing amino nitrogen. The action of the endoenzymes was stronger than that of the exoenzymes, in the case of the fungi, but, in the case of the actinomycetes, the reverse held true in most instances; this is probably due to the fact that the treated mycelium containing the endoenzymes of the actinomycetes was added in much smaller quantities than that of the fungi since the growth of the former is very scant. It is interesting to note one thing in the above table, namely that *P. chrysogenum* and *Act.* sp. 101, which were found to produce more amino nitrogen than ammonia when grown in a peptone solution (Waksman, 1918), produced enzymes, which have a strong action upon the proteins, while the other organisms, which produced more ammonia than amino nitrogen in the peptone solution, gave rise to much weaker enzymes; this fact may throw some light upon the problem of protein decomposition by microörganisms.

It was pointed out by Kendall and his associates (1915) that the sugar content of the medium has a decided influence upon the production of proteolytic enzymes by bacteria. To see whether the same thing holds true with fungi, the enzymes obtained from *A. niger* grown for fifteen days on the Peptone-Czapek solution containing 0, 1, 3, 5 and 20 per cent of sugar were used.

It is seen from table 2 that the sugar content of the medium has no decided influence upon the production of proteolytic enzymes by *A. niger*. Where sugar was present in the original medium just as much protein is split by both enzymes and, in some cases, even more than by the enzymes of the organism grown on the sugar free medium. The liquefaction of gelatin by the enzymes obtained from the above cultures did not appear to be affected by the presence of sugar in the original medium. The bacteria seem to behave differently from the fungi in this respect.

It was found advisable to determine first the proper reaction which would be optimum for the activities of the enzymes and also the proper age of the cultures, when the activities of the exoenzymes would be at a maximum. Several organisms were

grown on Czapek's and Peptone-Czapek solution and the filtrates only used for experiment.

The results brought out in the above experiment show a distinct difference between the activities of microbial and animal proteolytic enzymes. In most instances the substratum neutral to litmus contained a larger quantity of amino nitrogen; at least the more alkaline reaction did not prove more favorable for the enzyme activities of microörganisms than the more acid reaction. This would seem to point to several conclusions: either the organisms produce a mixture of two enzymes which are peptic and ereptic in nature, or, if the enzyme is of a tryptic

TABLE 2

Influence of sugar content of the medium upon the proteolytic activities of the exo- and endoenzymes of A. niger.

SUGAR CONTENT OF MEDIUM	MILLIGRAMS OF $NH_2=N$ PER 100 CC. OF SOLUTION			
	1 per cent peptone		1 per cent casein	
	Exoenzyme	Endoenzyme	Exoenzyme	Endoenzyme
Control	21.32	20.30	7.28	6.20
0	45.24	41.84	45.00	39.44
1 per cent	46.40	44.72	54.56	54.00
3 per cent	47.60	50.56	43.44	54.56
5 per cent	38.86	45.80	25.38	55.06
20 per cent	44.24	52.78	38.28	56.32
Trypsin: 200 mgm	46.68		56.56	

nature, it is much less sensitive to an acid reaction than animal trypsins are. Finally, the enzymes produced by the microorganisms studied may be of a nature entirely different from that of the animal proteolytic enzymes, and may be comparable to those found by Vines (1900–1910) in plants; the range of their optimum reaction is certainly greater than that of corresponding animal enzymes. *A. niger* and *A. fuscus*, both closely related species, produced, in the case of casein, less amino nitrogen with the more acid reaction, and the same thing held true for the enzymes of *A. niger* and *Act.* sp. 101 grown on the peptone-containing medium.

When the activities of the enzymes are compared with regard to the age of the culture, we find that the five-day-old cultures gave, on the average, a higher amino nitrogen production from both peptone and casein, and with both reactions. This is not fully in accord with the idea of Dox (1910) that in the older cultures the enzymes are excreted into the substratum, and the liquid therefore becomes richer in enzymes. It is likely however that the weakness of the enzymes in the older cultures may be due to an accumulation of acids or other waste products in the media, which might influence injuriously the activities of the enzymes.

That the enzymes produced by the organisms studied are not merely pepsins can be seen by glancing at table 3. The peptone was split just as well as the casein, giving amino-acids and other simple amino-nitrogen-containing compounds, the identity of which has not been established with certainty.

The culture medium, on which the organisms were grown, seems to have a decided influence upon the amount of enzymes present in the medium. The peptone-containing medium gave in all instances a much stronger proteolytic action than the peptone-free medium; but even on the peptone-free media proteolytic enzymes were also produced. This points to the fact that the variation in enzyme production by microörganisms on different media is not of a qualitative but of a quantitative character, as has already been demonstrated by other investigators.

Small quantities of ammonia amounting to a few milligrams of nitrogen per 50 cc. of solution were found in the substrata inoculated with the enzymes; this is probably due to the action of the desamidasing enzymes, as was shown by Shibata (1902), Pringsheim (1908), and Dox (1910).

It was thought advisable to repeat the study of the influence of reaction upon the enzyme activities of microörganisms. One per cent peptone solutions were made up neutral to phenolphthalein, and divided into three portions; one portion was left at that reaction and is termed "alkaline;" to a second portion 3 cc. of 0.1N HCl were added for each gram of casein or peptone,

this solution being termed "neutral;" to the third portion 5 cc. of 0.1N HCl were added for each gram of casein and peptone, this solution being termed "acid." *A. niger* and *A. ochraceus* grown for fourteen days on Czapek's and Peptone-Czapek solutions were used. The enzyme cultures were incubated for four days.

TABLE 3

The influence of composition of medium and reaction upon the production of amino nitrogen from proteins by the exoenzymes of microörganisms

ORGANISMS USED	CULTURE MEDIUM	AGE OF CULTURE	MILLIGRAMS OF NH_2=N PER 100 CC. OF SOLUTION			
			1 per cent peptone		1 per cent casein	
			Phenol-phthalein*	Litmus*	Phenol-phthalein	Litmus
		days				
Control			21.24	21.24	7.26	7.26
A. niger	Czapek's	5	21.46	34.16	23.78	26.10
A. niger	Czapek's	14	21.30	22.20	38.88	19.44
A. ochraceus	Czapek's	5	21.88	33.64	30.60	34.80
A. ochraceus	Czapek's	14	22.62	23.78	9.86	16.82
A. ochraceus	Peptone-Czapek	14	39.44	45.24	41.76	50.76
A. fuscus	Czapek's	14	21.40	23.56	20.30	7.54
A. fuscus	Peptone-Czapek	14	41.56	50.76		
P. chrysogenum	Peptone-Czapek	8	28.12	62.48	19.71	19.71
Citr. glaber	Czapek's	5	39.44	33.06	31.90	39.44
Act. californicus	Czapek's	15	33.06	47.56	31.32	52.20
Act. sp. 101	Czapek's	15	23.78	27.04	7.54	7.40
Act. sp. 101	Peptone-Czapek	15	33.06	30.16	45.82	45.24
Act. sp. 101	Peptone-Czapek	38	70.38	52.17	75.02	53.50
Act. violaceus-ruber	Czapek's	90	28.71	28.71	17.25	23.64
Trypsin: 200 mgm.			60.90	45.24	81.20	56.26
Pepsin: 200 mgm.			22.62	26.68	13.92	39.44

* Phenolphthalein = neutral to phenolphthalein, litmus = neutral to litmus.

The data presented in table 4 confirm the previous observations concerning the nature of the exoenzymes produced by microörganisms. In this case also the peptone containing media contained a more active enzyme or a stronger concentration of proteolytic enzymes than the non-protein media. As to the

influence of reaction, the highest production of amino nitrogen took place, in most instances, when the reaction was almost neutral to litmus. All the three reactions proved to be more or less favorable to the enzyme action, but the medium reaction which was not too acid nor too alkaline gave the best results. The enzymes of the fungi studied seem to have a wider range of reaction than the animal enzymes.

To obtain further information on the influence of the reaction upon the activities of the enzymes studied and also to throw some light upon the possibility of their being a mixture of two

TABLE 4

Influence of reaction upon the enzyme activities of microörganisms

ORGANISMS USED	CULTURE MEDIUM	REACTION OF SOLUTION	MILLIGRAMS OF $NH_2=N$ PER 100 CC. OF SOLUTION	
			1 per cent peptone	1 per cent casein
Control			21.40	7.28
A. niger	Czapek's	Alkaline	23.00	12.40
A. niger	Czapek's	Neutral	23.60	25.60
A. niger	Czapek's	Acid	21.44	13.44
A. niger	Peptone-Czapek	Alkaline	37.18	19.44
A. niger	Peptone-Czapek	Neutral	64.90	39.56
A. niger	Peptone-Czapek	Acid	25.00	24.80
A. ochraceus	Czapek's	Alkaline	24.92	53.34
A. ochraceus	Czapek's	Neutral	30.70	38.96
A. ochraceus	Czapek's	Acid	21.26	27.36
A. ochraceus	Peptone-Czapek	Alkaline	44.88	69.32
A. ochraceus	Peptone-Czapek	Neutral	55.14	73.76
A. ochraceus	Peptone-Czapek	Acid	32.50	74.84

kinds of enzymes, one acting best in a slightly acid and the other in an alkaline reaction, the following experiment was made. Exo- and endoenzymes of *A. niger* were obtained by the previously described methods from eight-day-old cultures grown on the Peptone-Czapek solution. Twenty cubic centimeters of the filtrate containing the exoenzyme and 800 mgm. of the mycelium containing the endoenzyme were added to 1 per cent peptone and casein solutions. The reaction of the substrata was made neutral to litmus, which was found in the previous experiments to be the best reaction for the activity of these enzymes. The

substrata plus enzymes were incubated for forty-eight hours at 37°C. At the end of that period, the amino nitrogen content of the cultures was determined; one set of cultures was then made neutral to phenolphthalein (no. 2 in table 5), while the others were left untouched (no. 1 in the table); the cultures were then further incubated for forty-eight hours and amino nitrogen determined. The results are given in table 5.

It is apparent that, if the proteolytic enzymes of *A. niger* are a mixture of several enzymes, the possibility of the existence of one enzyme which may act in a reaction optimum for tryptic activities is excluded, unless that enzyme was destroyed while the

TABLE 5

Influence of reaction upon the enzyme activities of A. niger.

REACTION	PERIOD OF INCUBATION	MILLIGRAMS OF $NH_2=N$ PER 100 CC. OF SOLUTION			
		1 per cent peptone		1 per cent casein	
		Exoenzyme	Endoenzyme	Exoenzyme	Endoenzyme
	hours				
Control		21.36	20.24	7.24	6.30
1. Neutral to litmus	48	30.80	50.40	16.80	39.20
1. Neutral to litmus	96	31.08	56.00	17.92	56.40
2. Neutral to phenolphthalein	48	30.64	50.54	16.52	38.46
2. Neutral to phenolphthalein	96	30.08	49.92	15.40	37.58

culture was incubated for the first forty-eight hours; which is very doubtful, since the medium itself, where the enzyme is produced, is very acid. The H-ion concentration of the filtrate of the eight-day-old culture of *A. niger* grown on the Peptone-Czapek medium was often as high as pH = 2.0. The fact is that the neutralization of the substratum to phenolphthalein, after forty-eight hours of incubation at a neutral reaction, checked the further activity of the enzyme, as is seen in table 5, where the amount of amino nitrogen is found to increase in the neutral culture, while the culture made alkaline gave no further increase; the slight decrease found is due to the dilution of the substratum through the addition of the alkali for change of re-

action. Long and Hull (1917) have recently shown that trypsin acts on fibrin and fibrin-peptone most energetically at an H-ion concentration between 10^{-8} and 5.10^{-9}, and on casein at 3.10^{-6} to 5.10^{-7}. The enzyme of *A. niger*, although acting on casein at a reaction similar to the one found to be optimum for trypsin

TABLE 6

Influence of age of culture upon the activities of proteolytic exo- and endoenzymes of microörganisms

ORGANISMS USED	CULTURE MEDIUM	INCUBATION	MILLIGRAMS OF NH_2=N PER 100 CC. OF SOLUTION			
			1 per cent peptone		1 per cent casein	
			Exoenzyme	Endoenzyme	Exoenzyme	Endoenzyme
		days				
Control			21.24	20.35	7.18	6.22
A. niger	Czapek's	3	26.30	53.41	12.27	90.60
A. niger	Czapek's	10	21.64	36.42	11.38	33.00
A. niger	Czapek's	18	21.84	39.76	10.64	28.00
A. niger	Czapek's	35	21.76	34.77	7.84	15.39
A. niger	Peptone-Czapek	3	28.67	84.82	19.89	125.77
A. niger	Peptone-Czapek	10	24.26	59.75	25.71	80.80
A. niger	Peptone-Czapek	18	29.12	64.40	21.28	92.40
A. niger	Peptone-Czapek	35	46.74	43.89	18.24	22.33
A. ochraceus	Czapek's	3	26.32	47.97	19.31	38.02
A. ochraceus	Czapek's	10	31.86	68.28	43.87	73.97
A. ochraceus	Czapek's	18	34.72	71.68	50.40	82.88
A. ochraceus	Czapek's	35	22.46	57.00	20.32	76.95
A. ochraceus	Peptone-Czapek	3	26.91	49.14	53.24	55.58
A. ochraceus	Peptone-Czapek	10	62.59	80.80	58.03	79.66
A. ochraceus	Peptone-Czapek	18	42.56	93.52	64.40	115.92
A. ochraceus	Peptone-Czapek	22	62.70	64.26	68.40	74.10

by Long and Hull, acts on peptone and on fibrin (as will be seen later) at a higher H-ion concentration.

To throw more light upon the influence of the age of the culture on the proteolytic activities of both kinds of enzymes, it was thought advisable to grow *A. niger* and *A. ochraceus* on both media, in a large number of flasks. At the end of the proper incubation period, single flasks containing each organism were taken out of the incubator and filtered. The mycelium was

treated by the acetone method, and 0.8 gram of the dried mycelium was used in each case. The organisms were grown for 3, 10, 18, 35 days at 28°C.; the enzyme cultures were in all cases incubated for four days at 37°C. The amino nitrogen produced due to the activities of the enzymes was determined as usual, and results calculated back to 100 cc. of the substrata.

When we compare the influence of the medium upon the activities of the enzymes, we see clearly that the presence of peptone in the medium greatly increases the activities of the enzymes, both extra- and intracellular, in their action on peptone and casein, as shown in table 7; this effect is observed with all the periods of incubation studied. It is remarkable that in most cases *A. niger* produced the strongest enzymes in the first period of incubation, namely in three days, particularly when grown on the peptone free medium. There is a more or less steady decrease in the activities of the enzymes with the increase of the incubation period, and the enzymes obtained from the organism grown on the peptone medium particularly the exoenzymes, show greater irregularities in this respect.

In the case of the enzyme obtained from *A. ochraceus*, we find that the activities of the enzymes increased from the third till the eighteenth day of incubation of the organism, further incubation giving a decrease in the activities of the enzymes. Only in two cases is an increase in the activities of the enzymes found after the growth of the organism was continued for a period greater than eighteen days, namely in the case of the exoenzyme of that organism growing on peptone. The difference in the activities of the two organisms may be explained on the following assumptions: *A. niger* grows very rapidly and produces a great deal of acid, mostly oxalic and citric, in both media; while *A. ochraceus* grows comparatively slowly and produces no acid at all or very little. The rapid growth of *A. niger* will result in an early production of strongly acting enzymes; the acids produced by this organism may act injuriously upon the enzymes and their activity will therefore be strongest in the earliest period of incubation. The slower growth of *A. ochraceus* will result in a lack of strongly acting enzymes in the early stages of the growth

of the organism, but their activity will increase with the age of the organism, since no acids or other harmful agents are produced, till the organism begins to autolyze, and then the further production of the enzymes may be affected by the products of autolysis. The difference in behavior of the enzymes of *A. niger* grown on the peptone medium may be explained by the fact that the peptone and its decomposition products present in the medium may exert a buffer action upon the acids of the medium thus decreasing their injurious effect.

TABLE 7

Influence of incubation of enzymes upon the amino nitrogen produced

ORGANISMS USED	CULTURE MEDIUM	AGE OF CULTURE	INCUBATION	MILLIGRAMS OF $NH_2=N$ PER 100 CC. OF SOLUTION			
				1 per cent peptone		1 per cent casein	
				Exoenzyme	Endoenzyme	Exoenzyme	Endoenzyme
		days	*hours*				
Control				21.46	20.30	7.30	6.28
A. niger	Peptone-Czapek	10	2		24.34		15.85
A. niger	Peptone-Czapek	10	4		32.26		21.51
A. niger	Peptone-Czapek	10	18		42.81		34.47
A. niger	Peptone-Czapek	10	23		46.90		43.51
A. niger	Peptone-Czapek	10	40		52.63		47.09
A. niger	Peptone-Czapek	10	88		54.90		50.51
A. niger	Peptone-Czapek	10	112		61.94		58.07
A. niger	Peptone-Czapek	10	160		63.60		
A. niger	Peptone-Czapek	18	2		28.30		17.55
A. niger	Peptone-Czapek	18	4		29.43		22.64
A. niger	Peptone-Czapek	18	18		40.03		44.48
A. niger	Peptone-Czapek	18	23		42.38		50.98
A. niger	Peptone-Czapek	18	40		52.08		59.28
A. niger	Peptone-Czapek	18	88		59.72		74.10
A. niger	Peptone-Czapek	18	112		61.94		76.31
A. ochraceus	Peptone-Czapek	10	2	31.13	30.00	32.26	30.55
A. ochraceus	Peptone-Czapek	10	4	33.96	33.39	33.96	35.66
A. ochraceus	Peptone-Czapek	10	18	41.14	41.14	46.15	57.88
A. ochraceus	Peptone-Czapek	10	23	43.51	45.20	51.98	74.02
A. ochraceus	Peptone-Czapek	10	40	47.64	51.52	60.94	84.76
A. ochraceus	Peptone-Czapek	10	88		53.80	62.04	
A. ochraceus	Peptone-Czapek	10	112		56.96	63.60	
A. ochraceus	Peptone-Czapek	10	160		67.56	70.38	

The following experiment was made with the purpose of demonstrating the influence of the incubation of the enzyme cultures upon the amino nitrogen production, the reaction of the substrata being neutral to litmus.

When the velocities of reaction of the different enzymes are compared, one finds in all cases an initial rapid increase in the amounts of amino nitrogen produced, followed by a gradual decrease in velocity.

The enzyme cultures were incubated in all previous experiments at 37°C. which was found to be the optimum temperature for the action of the proteolytic enzymes derived from animal tissues. But since the action of the enzymes of microörganisms differs so much from that of the animal enzymes, it was thought advisable to study the influence of incubation upon the activities of the enzymes used in this work. Both exo- and endoenzymes of *A. niger* grown for eight days on the Peptone-Czapek medium were used. Twenty cubic centimeters of the exoenzyme containing filtrate and 0.8 gram of the endoenzyme containing mycelium were added to peptone and casein solutions so as to make the concentration of the substrata just 1 per cent; the reaction was made neutral to litmus and the proper disinfectants were added. The cultures were incubated at 12°, 23°, 29°, 34°, and 39°C. for forty-eight hours; at the end of that period the amino nitrogen was determined in all the solutions.

The facts brought out in table 8 tend to show that the range of temperature optimum for the action of the enzymes of *A. niger* is comparatively great. Even at as low a temperature as 12°C. the splitting of the proteins still took place, the action increasing with the increase in temperature. The optimum was reached at 29° to 34°C., followed by a drop in activity at 39°C. We would naturally expect that an organism, whose temperature optimum lies below the optimum for the enzymes of the warm blooded animals, should produce enzymes which will act best at somewhat lower temperatures.

The question as to the nature of the enzymes of microörganisms cannot be definitely answered as yet from the previous experiments. It has been proven that both exo- and endo-

enzymes of the microörganisms used can decompose peptone and casein, but this does not tell us with what group of enzymes these should be classified, whether with the trypsins or with the erepsins, both of which, when obtained from animals, split peptone and casein. But these are active only in alkaline solutions, while the enzymes of the microörganisms studied act best in neutral and even slightly acid media. This would seem to indicate that the latter do not belong to the same class of enzymes as those obtained from animal tissues. Went (1901), Butkewitch (1903), Saito (1903), and Franceschelli (1915) claimed that we are dealing with trypsins. The work of Dox (1910)

TABLE 8

Influence of temperature of incubation upon the activities of the proteolytic enzymes of A. niger

TEMPERATURE	MILLIGRAMS OF $NH_2=N$ PER 100 CC. OF SOLUTION			
	1 per cent peptone		1 per cent casein	
	Exoenzyme	Endoenzyme	Exoenzyme	Endoenzyme
deg. C.				
Control	21.46	20.40	7.24	6.18
12	29.18	33.60	11.24	11.80
23	29.46	48.72	14.00	20.72
29	33.04	56.00	16.80	56.28
34	34.16	69.44	21.12	56.00
39	30.24	45.36	17.36	39.20

Reed and Stahl (1911), Berman and Rettger (1916) and others would lead us to think that we are dealing here with erepsins, which may be very close to animal erepsin. The work of Vines (1900–1910) on the enzymes of plants and those of *Aspergillus oryzae* and the studies of Hagem (1910) tend to prove that we have here a mixture of peptases and ereptases more closely allied to the corresponding plant enzymes than to the animal enzymes. The fact that certain investigators have obtained the liquefaction of gelatin, which resulted in the production of peptone, by the enzyme of *A. niger* leads us to think that this organism produces enzymes which, besides being of an ereptic nature, are also tryptic or peptic in nature. The work of Malfitano (1900) and

of Steffens (1900) tend to show that the enzymes of molds are entirely different from animal enzymes.

Erepsin, as was shown by Cohnheim (1901), Fränkel (1916) and other investigators, decomposes not only peptone but also casein, so that the two substances used in the previous experiments cannot give us any differentiation between erepsin and other enzymes which could also decompose true proteins. Merck's fibrin and crystalline egg-albumen were therefore used

TABLE 9

Decomposition of fibrin and crystalline egg-albumen by enzymes of microörganisms

ORGANISMS USED	CULTURE MEDIUM	AGE OF CULTURE	MILLIGRAMS OF NH_2=N PER 100 CC. OF SOLUTION					
			1 per cent fibrin			1 per cent egg-albumen*		
			Exoenzyme		Endoenzyme	Exoenzyme*		Endoenzyme*
			Control	Enzyme	Control=0	Control	Enzyme	Control = 7.4
		days						
A. niger........	Czapek's	35	1.28	19.92	21.05	8.02	18.21	22.76
A. niger........	Peptone-Czapek	18			22.76			46.66
A. niger........	Peptone-Czapek	35	11.10	21.05	6.26	13.16	27.31	25.61
A. ochraceus....	Czapek's	10	1.14	46.66	14.23	8.16	29.59	34.14
A. ochraceus....	Czapek's	35	1.64	11.99	25.04	8.28	11.38	44.38
A. ochraceus....	Peptone-Czapek	10	10.04	40.40	82.51	12.48	32.43	38.69
A. ochraceus....	Peptone-Czapek	35	12.16	27.31	31.30	14.26	40.40	30.16
P. chrysogenum.	Czapek's	35	2.56	52.92		8.64	34.71	
Act. griseus.....	Czapek's	90	0	36.42		7.96	23.33	

* The presence of amino nitrogen in the crystalline egg-albumen may be due either to the end group of the protein molecule, or to such product combined with a small quantity of ammonia, remaining as a contamination of the protein in the form of $(NH_4)_2SO_4$.

for the next experiment. The fibrin was introduced, in small pieces into flasks containing 100 cc. of water so as to make one per cent of the liquid, after the proper enzyme has been added. The crystalline egg-albumen was also made up as a 1 per cent solution in water. The exo- and endoenzymes of *A. niger*, *A. ochraceus*, *P. chrysogenum*, and *Act. griseus* were used for this experiment.

As is seen from table 9, such true proteins as fibrin and crystalline egg-albumen are also decomposed by the enzymes of the

microörganisms studied; the different enzymes behaved somewhat differently in this case, the largest amount of amino nitrogen being obtained from fibrin by the exoenzymes of *P. chrysogenum* and by the endoenzymes of *A. ochraceus*.

The digestion of fibrin and crystalline egg-albumen is shown to be positive, hence the enzymes of the microörganisms cannot be erepsins or only ereptic in nature; although it is possible that other organisms behave in an entirely different way from those studied in this paper.

It still remained to find out whether the enzymes are tryptic in nature. Otsuka (1916) claimed that trypsin retains its activity after filtration through a Chamberland filter, but erepsin becomes inactive. The filtrate of a culture of *A. niger* grown for eight days on the Peptone-Czapek solution was filtered through a no. 6 Pasteur-Chamberland filter, and both filtered and unfiltered portions were compared with the untreated liquid as to their enzymatic power. The tests were performed in the ordinary way, by adding 20 cc. of each fluid to 80 cc. of peptone and casein solutions, so as to make them of 1 per cent concentration; these were incubated for forty-eight hours at 37°C. and amino nitrogen determined.

As is seen from table 10, the filtering of the enzyme culture through a porcelain filter results only in a very slight diminution in the activity of the enzymes, which may be due to a mere mechanical retention by certain colloidal particles of a small quantity of the enzyme. If the results of Otsaka hold true for erepsin, the enzymes of the microörganisms studied do not seem to be erepsins.

Robertson (1907) pointed out the fact that when a saturated solution of safranin is added to a neutral or faintly alkaline solution of trypsin, a flocculent precipitate is formed which contains the most active constituents of the trypsin. When this precipitate is added to a solution of a protein, the latter will be decomposed very rapidly, while the filtrate left, after the safranin-trypsin precipitate is removed, is almost inactive. This behavior of the safranin toward the trypsin in solution can be utilized for the testing of the tryptic nature of the exoenzymes of

microörganisms. Two liters of the filtrate from an eight-day-old culture of *A. niger* grown on the Peptone-Czapek solution were used for this work; this filtrate was strongly acid due to the large quantities of oxalic and citric acid produced by the organism. One liter of the fluid was left acid and the other was neutralized to phenolphthalein by means of N NaOH. To each liter of the fluid 20 cc. of a saturated solution of safranin were added. A flocculent colored precipitate appeared in both instances, but was much heavier in the neutral solution. The precipitates were allowed to settle for twenty-four hours, then filtered off, washed with alcohol, and dried over sulfuric acid. The precipitate from the liter of medium left acid weighed 60 mgm. and that from the liter of neutral medium weighed 500

TABLE 10

Influence of filtration through a porcelain filter upon the action of exoenzymes of A. niger

	MILLIGRAMS OF $NH_2=N$ PER 100 CC. OF SOLUTION					
	1 per cent peptone			1 per cent casein		
	Untreated culture	Filtered portion	Unfiltered portion	Untreated culture	Filtered portion	Unfiltered portion
At start	21.38	21.30	21.36	7.44	7.24	7.42
After 48 hours	28.62	25.16	27.32	15.77	12.30	13.88

mgm. The precipitates and the filtrates left, after their removal were now tested for their proteolytic activities. About one-half of each precipitate and 20 cc. of the filtrate left were used as the sources of enzymes; as substrata 100 cc. of 1 per cent peptone and casein solutions were used. The cultures were incubated for forty-eight hours at 37°C.

It is seen from table 11 that the safranin does not precipitate the enzyme from the culture medium. One liter of medium, or just 50 times as much as is ordinarily used for the inoculation of 100 cc. of the 1 per cent substratum, did not give, on precipitation with safranin, enough enzyme to decompose as much of the protein, as 20 cc. of the solution usually does. We might therefore conclude that the enzyme is not of the nature of trypsin.

The small quantity of substratum decomposed by the safranin precipitate might be merely due to the fact that some of the enzyme was carried down mechanically by the safranin precipitate, and not in the form of a salt such as the color-base safranin is supposed to form with the acid-like trypsin. The filtrates from the safranin precipitate were weaker in their enzymatic action than the normal solution obtained by filtering the original culture of the organism. This may be due to several reasons: first, the filtrate from the safranin precipitate was slightly diluted by the addition of the safranin solution; second, some of the enzyme might have been carried down by the precipitate; and third, the safranin itself may have had some injurious effect upon the enzyme. There does not seem to be any doubt that

TABLE 11

The action of safranin as a precipitating agent for the exoenzymes of A.niger

	MILLIGRAMS OF $NH_2=N$ PER 100 CC. OF SOLUTION					
	1 per cent peptone			I per cent casein		
	Normal exoenzyme	Precipitate	Filtrate	Normal exoenzyme	Precipitate	Filtrate
Control..............	21.40	20.24	21.12	7.36	6.12	7.20
Acid.................		22.96	28.40		6.72	11.76
Neutral..............	34.16	31.92	29.12	29.68	8.96	12.88

the enzymes of many microörganisms can attack true proteins. What, then, is the true nature of the enzymes? They cannot be similar to the animal trypsins, because they act best in a neutral and even slightly acid medium, are not precipitated by safranin, and act best at lower temperatures. They cannot be pepsins alone, because they decompose peptones and the splitting of casein and other proteins goes further than the peptone stage. The only thing that could be suggested is that these enzymes should not be classified with the animal proteolytic enzymes at all. If anything, they approach nearer the plant enzymes and they are either tryptases similar to the animal trypsins, but differentiated from them by certain characteristics; or they are a

mixture of peptases and ereptases, as Vines (1900–1910) suggested for plants.

Corper and Sweany (1917) claimed that the tubercle bacilli possess a tryptic-like enzyme capable of splitting proteins in alkaline solutions, a weak pepsin-like enzyme capable of splitting proteins in acid solutions, and an erepsin-like enzyme capable of decomposing peptones in acid solutions. As a matter of fact, the same enzymatic activities are found to hold true also for the molds and the actinomycetes studied, and the explanation of the co-existence of the three different enzymes in the same organism can be used here also as an explanation of the peculiar behavior of the enzymes of the microörganisms studied. This would simplify greatly the question as to the nature of the enzymes of microörganisms, but the explanation would be of doubtful importance in explaining the true nature of the enzymes of the organisms studied. Before we are able actually to separate the different enzymes, the only assumption that can be made is that the proteolytic enzymes of microörganisms behave in a different manner from animal enzymes and should be placed in a class by themselves.

SUMMARY

1. The proteolytic enzymes contained in the fungi studied appear to differ from known proteolytic enzymes of animal origin in the following particulars:

a. Their range of optimum reaction is greater; a reaction neutral to litmus was found to be the optimum one for the activities of the majority of the enzymes studied.

b. The temperature optimum is somewhat lower.

c. Although acting best in a neutral or sometimes in a slightly acid medium they differ from animal trypsin in not being precipitated by safranin.

d. The exoenzymes can pass through a Pasteur-Chamberland filter.

2. The sugar content of the medium has no influence upon the production of proteolytic exo- and endoenzymes of *A. niger*.

3. Both exo- and endoenzymes are produced by microörganisms on protein-containing and protein-free media, but the activities of the enzymes from organisms grown on the media containing proteins are greater than those obtained from organisms grown on the protein-free media.

4. The age of the culture, at which the most active enzymes are obtained, depends on the organism itself, its rapidity of growth, and the nature of the waste products produced in the medium.

5. Fibrin and crystalline egg-albumen are decomposed by both the exo- and endoenzymes of the organisms used.

6. Small quantities of ammonia were found to be produced in the decomposition of peptone and casein by the proteolytic enzymes of the microörganisms studied. This fact indicates the probable presence of desamidases among the enzymes produced.

The writer wishes to express his most sincere thanks to Prof. T. B. Robertson and to Dr. C. L. A. Schmidt for many helpful suggestions in carrying out this problem, and to Prof. C. B. Lipman for reading the manuscript.

REFERENCES

Abderhalden, E., and Pringsheim, H. 1910 Ztsch. f. physiol. Chem. **65**, 180.
Berman, N., and Rettger, L. F. 1916 J. Bact., **1**, 537.
Butkewitsch, W. 1903 Jahr. Wiss. Bot., **38**, 147.
Cohnheim, O. 1901 Ztschr. f. physiol. Chem., **33**, 451.
Corper, H. J., and Sweany, H. C. 1917 J. Biol. Chem., **29**, xxi Proc.
Dox, A. W. 1910 Bull. 120, Bureau Anim. Ind., U. S. Dept. Agr.
Franceschelli, D. 1915 Centralbl. f. Bacteriol., II Abt., **43**, 305.
Fränkel, E. M. 1916 J. Biol. Chem., **26**, 31.
Fuhrman, F. 1907 Forlesungen über Bakterienenzyme, Jena.
Hagem, O. 1910 Vidensk. Selsk., Math. Naturw. Klasse, **10**, 1.
Hopkins, F. G., and Pinkus, S. N. 1898 J. Physiol., **23**, 130.
Kendall, A. I., and Walker, A. W. 1915 J. Infect. Dis., **17**, 442.
Long, J. H., and Hull, M. 1917 J. Am. Chem. Soc., **39**, 1051.
Malfitano, G. 1900 An. de l' Inst. Pasteur, **14**, 60, 420.
Otsuka, I. 1916 Acta Scholoe Med. Kyoto, **1**, 199; ref. Physiol. Abstr. **2**, 1917, 15.
Pringsheim, H. 1908 Biochem. Ztschr., **12**, 15.
Reed, H. S., and Stahl, H. S. 1911 J. Biol. Chem., **10**, 109.
Saito, 1903 Bot. Magaz. Tokyo, **18**, 75.

Scales, F. M. 1914 J. Biol. Chem., **19**, 459.
Shibata, K. 1904 Beitr. Chem. Physiol. u. Pathol., **5**, 384.
Steffens 1900 Inaug. Diss., Erlangen.
Thom, C. 1916 J. Agric. Research, **7**, 1.
Van Slyke, D. D. 1911 J. Biol. Chem., **9**, 185; **16**, 121.
Vínes, S. H. 1900–1910 Ann. Bot. **17**, 237, **18**, 289, **19**, 171, **20**, 113, **23**, 17, **24**, 213.
Waksman, S. A. 1916 Soil. Sc. **2**, 102.
Waksman, S. A. 1918 J. Bact. **3**, 475.
Wehmer, C. 1907 Lafar's Handbuch technischer Mykologie **4**, 239
Went, F. A. F. C. 1901 Jahr. Wiss. Bot., **36**, 611.

SOME OBSERVATIONS ON THE EFFICIENCY OF THE PRESENT STANDARD AGAR FOR THE ESTIMATION OF BACTERIA IN MILK

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On receipt of the last report of the Committee on Standard Methods[1] the workers in this laboratory noted with surprise and also with some skepticism that the content of nutrient substances in the standard agar had been reduced still further. We accepted the revision of methods, however, in their entirety and have been adhering to the new standards ever since but with an attitude somewhat more critical than heretofore. Whether it was due to this critical attitude or whether it was on account of the change in the composition of the standard agar, it is a fact that we soon noticed inaccuracies and inconsistencies in some of our results which were too important to be overlooked.

The chief difficulty which we have encountered, and for which we are constrained to blame the present standard agar, consists in the failure of the counts made from the different dilutions of the same sample to check with each other. It was not infrequently noticed, for example, that the plates from the 1–100 dilution would give the sample a total count many times greater than those from the 1–1000 dilution. Very often it would be quite impossible to count the former while the latter would show fewer than the minimum of 20 colonies. This sort of a situation could only leave the worker the choice of estimating the number of colonies on the very much overcrowded 1–100 plates or of reporting the number indicated by the obviously unreliable 1–1000 plates.

[1] Provisional Report of the Committee on Standard Methods of Bacteriological Analysis of Milk, 1916, Am. Jour. Pub. Health, 6, 1315.

It was always observed that when the experience just described occurred it would be found that the 1–100 plates contained large numbers of very small round colonies resembling streptococcus colonies but differing from the latter in being whiter and more opaque. These tiny colonies ranged in size from that of a pin-head, to the merest points. The recognition of the latter as colonies, even with the aid of the standard lens, was practically impossible. It was only the gradation in size that indicated that they were colonies and not specks in the medium. These very minute colonies appeared on the 1–1000 plates only very

TABLE 1

TOTAL BACTERIA PER CUBIC CENTIMETER INDICATED BY 1-100 COUNTS	TOTAL BACTERIA PER CUBIC CENTIMETER INDICATED BY 1-1000 COUNTS
44,000	16,000
15,000	5,000
39,000	4,000
74,000	28,000
46,000	3,500
19,000	7,000
90,000	1,000
28,000	9,000
18,000	3,000
20,000	8,500
11,000	1,000
16,000	1,500
43,000	10,000
29,000	8,500

rarely, and when they did they often were found on only one of the duplicates. Their appearance always accompanied a wide difference in the counts of the duplicate plates. Table 1 gives the counts of fourteen samples selected from our records.

The inconsistencies described seemed very mysterious at first but on consideration it was seen that the explanation must lie in the difference in composition between the nutrient medium in the 1–100 plates and that in the 1–1000 plates. Inasmuch as the same nutrient agar was used in each this difference could only arise from the amount of milk introduced in the sample. One one hundredth cubic centimeter of milk introduced into a

10 cc. quantity of the standard agar so enriched the latter that certain organisms could multiply to the extent of producing visible colonies while these organisms could not do so when only 0.001 cc. of milk was added. In other words, the addition to, or elimination from, a tube of agar of 0.009 cc. of milk was sufficient to change very materially the growth supporting qualities of the medium. It appeared then that to keep the medium of constant composition in all dilutions it was only necessary to supply to the 1–1000 dilution and all higher dilutions enough milk to make up the deficiency. Ths was done approximately by making the higher dilutions in sterile 1–100 milk instead of in sterile water. Table 2 gives the results of a preliminary test to determine the efficiency of this improvement. In this initial test the 1–100 dilution was plated out in duplicate, but only one plate was made from each of the two 1–1000 dilutions. In a second test, the results of which are given in table 3, all three dilutions were plated out in duplicate. For convenience the columns showing the counts on the 1–100 plates are marked "C" and those showing the counts on the plates made from the 1–1000 dilutions in sterile water and sterile 1–100 milk are marked "M_1" and "M_2" respectively. The samples employed in these experiments were not selected samples. They were picked at random from those brought in for examination.

Several important points are noticed on examination of the figures in tables 2 and 3. In the first place it is observed that in many samples the M_2 count is considerably higher than the M_1 count and that the M_2 counts check better with the C counts than do the M_1 figures. This establishes the validity of the reasoning which was the basis of the experiment. The added milk enhances the growth supporting qualities of the medium. It is interesting also to note that in the majority of cases in which these differences appear the samples are those of pasteurized milk. In going through our records to get the figures given in table 1, samples were selected which best presented the inconsistencies mentioned. It was not noticed until afterwards that every sample was one of pasteurized milk.

Another fact, which is not indicated in the tables, but which is very apparent to the person doing the counting, is that in cases in which the very small colonies appear on both the M_1

TABLE 2

SAMPLE NUMBER	GRADE	TOTAL NUMBER OF BACTERIA PER CUBIC CENTIMETER INDICATED BY THE DIFFERENT PLATE COUNTS		
		C	M_1	M_2
1	Raw	3,300	0	2,000
2	Raw	14,000	4,000	14,000
3	Raw	90,000	130,000	110,000
4	Raw	2,600	2,000	5,000
5	Raw	42,000	22,000	29,000
6	Raw	1,500	0	1,000
7	Raw	2,500	0	3,000
8	Raw	3,400	7,000	3,000
9	Raw	1,400	0	1,000
10	Raw	5,600	5,000	9,000
11	Raw	86,000	140,000	150,000
12	Raw	200,000	150,000	490,000
13	Raw		150,000	210,000
14	Raw		140,000	260,000
15	Raw	17,000	13,000	36,000
16	Raw	9,400	14,000	17,000
17	Raw	38,000	27,000	34,000
18	Raw	8,600	8,000	5,000
19	Raw	12,000	16,000	10,000
20	Raw	66,000	70,000	72,000
21	Raw	95,000	95,000	120,000
22	Pasteurized	16,000	7,000	18,000
23	Pasteurized	9,700	4,000	10,000
24	Pasteurized	40,000	8,000	40,000
25	Pasteurized	43,000	9,000	37,000
26	Pasteurized	250,000	9,000	400,000
27	Pasteurized	250,000	0	9,000
28	Pasteurized	200,000	8,000	370,000
29	Pasteurized	26,000	0	39,000
30	Pasteurized	24,000	0	18,000
31	Pasteurized	12,000	3,000	8,000

and M_2 plates they are very much larger and more easily counted on the latter.

A qualitative examination of the minute colonies which were the source of the errors described above revealed the fact that

they were all composed of organisms of the lactic acid type. A large number were isolated and all were found to coagulate milk rapidly and to acidify glucose and lactose broths without the production of gas. Most of them were Gram negative bacilli, but diplococcus types were isolated from several samples.

TABLE 3

SAMPLE NUMBER	GRADE	TOTAL NUMBER OF BACTERIA PER CUBIC CENTIMETER INDICATED BY THE DIFFERENT PLATE COUNTS		
		C	M_1	M_2
1	Raw	26,000	31,000	56,000
2	Raw	25,000	10,000	28,000
3	Raw	47,000	58,000	57,000
4	Raw	28,000	32,000	31,000
5	Raw	27,000	23,000	33,000
6	Raw	7,500	8,000	7,000
7	Raw	21,000	18,000	30,000
8	Raw	2,700	6,500	5,000
9	Raw	8,200	8,000	8,500
10	Raw	5,600	6,000	9,500
11	Raw	45,000	36,000	43,000
12	Raw	7,300	10,000	11,000
13	Raw	30,000	54,000	38,000
14	Raw	3,400	3,500	4,500
15	Raw	11,000	16,000	15,000
16	Pasteurized	25,000	4,500	32,000
17	Pasteurized	120,000	46,000	160,000
18	Pasteurized	73,000	60,000	80,000
19	Pasteurized	73,000	110,000	160,000
20	Pasteurized	19,000	4,500	20,000
21	Pasteurized	46,000	26,000	37,000
22	Pasteurized	31,000	21,000	35,000
23	Pasteurized	8,000	5,000	15,000
24	Pasteurized	11,000	1,500	8,500
25	Pasteurized	74,000	72,000	84,000
26	Pasteurized	31,000	22,000	62,000
27	Pasteurized	52,000	53,000	63,000

A number of pure cultures of these organisms were tested on the standard agar, and in every case it was found that visible colonies would develop when the organisms were plated from an emulsion in 1–100 sterile milk and that they would almost invariably fail to do so when plated from an emulsion in water.

When colonies did appear on the latter plates their number was always small as compared with that on the plates made from the milk emulsion. The following is the result of one of several such tests. An emulsion was made in 1–100 sterile milk and 1 cc. of this emulsion mixed with 9 cc. of sterile water and 9 cc. of sterile 1–100 milk respectively. Plates made from the two last mixtures gave:

Water	(5) (3) (2)
1–100 milk	(89) (69)

The conclusions reached in this investigation are:

1. The present standard agar is not in itself a favorable medium for the growth of certain types of bacteria occurring in milk.

2. The small quantity of milk added to the medium in plating 1 cc. of a 1–100 dilution supplies the deficiency, but the amount added in plating the higher dilution does not do so.

3. More consistent results are obtained if the dilutions of the sample higher than 1–100 are made in sterile 1–100 milk instead of in sterile water.

STERILIZING MEASURED AMOUNTS OF WATER IN THE AUTOCLAVE[1]

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It is customary for bacteriologists to measure out specific amounts of water into flasks and then to sterilize the flasks and water in the autoclave. Water is known to be lost during the process of autoclaving but the amount lost is usually considered to be so small that the errors occurring in bacterial dilutions (from this source) are small in comparison to other errors made in plating and dilution work.

To ascertain just what the loss of water in autoclaving is and just what effect this loss would have on dilutions, several tests were made. Two of these tests are reported here.

The following tables give the results of one sterilization of water in bottles in the autoclave. In putting the water into the eight ounce bottles, used in these tests, the technic was as follows: Each bottle was weighed to the nearest decigram and 99 grams or 90 grams, as desired, in excess of the weight of the bottle was placed on the opposite pan of the balance. 99 cc. or 90 cc. aliquots of distilled water were measured out by means of a 100 cc. graduated cylinder and poured into each bottle. In no case was the amount of water poured in more than 0.35 of a gram away from that desired. Water was then taken out or added so that each bottle contained the weight desired.

The bottles were sterilized for fifteen minutes under 18 pounds pressure of live steam and then the pressure was reduced at the

[1] This report is part of the work done by the author in working out and classifying errors in the plate method of enumerating bacteria. The investigations on plate methods when completed will appear as a bulletin of the Indiana Agricultural Experiment Station.

rate of one pound per minute, the door being opened thirty-five minutes after it was first closed.

Test 1. Two 8 ounce saltmouth bottles containing 99 grams of water and two containing 90 grams of water.

All four bottles were plugged with absorbent cotton.

The results of this test are given in table 1.

TABLE 1

Loss of water from dilution bottles in autoclaving

BOTTLE NUMBER	WEIGHT OF H_2O PUT IN	WEIGHT AFTER STERILIZATION	LOSS IN WEIGHT	PER CENT OF WEIGHT LOST
	grams	*grams*	*grams*	
1	99	95.9	3.1	3.13
2	99	95.2	3.8	3.84
3	90	86.5	3.5	3.89
4	90	86.9	3.1	3.44
Average..................................			3.4	

Test 2. Fourteen 8 ounce saltmouth bottles containing 90 grams of water.

Seven were plugged with absorbent cotton and seven were left unplugged. They were set in the autoclave in sets of two. The two at the rear were numbered 1 and 2, and the two nearest the door 13 and 14. Even numbers denote bottles having no plugs. Experiment conducted as in test 1. Results are given in table 2.

It is evident from these tests that the errors due to loss of water in autoclaving are considerable even with plugs in place. When a number of successive dilutions are made these errors would accumulate so that high dilutions might vary as much as 20 per cent from the dilutions desired.

On the basis of these tests it might seem advisable to ascertain by tests the amount of water that is regularly lost from bottles of water sterilized in the auto-clave under specific conditions. In some cases the loss might be constant enough so that a fixed amount of water in excess of that desired could be added to allow for losses occurring during auto-claving.

TABLE 2

Effect of cotton plugs on loss of water in autoclaving

BOTTLE NUMBER	WEIGHT OF H_2O PUT IN	WEIGHT OF H_2O LOST	PER CENT OF H_2O LOST WITH PLUG	PER CENT OF H_2O LOST WITHOUT PLUG
	grams	*grams*		
1	90	2.2	2.44	
2	90	6.3		7.00
3	90	3.7	4.11	
4	90	8.3		9.22
5	90	3.0	3.33	
6	90	8.3		9.22
7	90	2.9	3.22	
8	90	3.7		4.11
9	90	3.0	3.33	
10	90	8.0		6.69
11	90	2.4	2.67	
12	90	7.6		8.44
13	90	2.5	2.78	
14	90	5.1		5.67
Average		4.8	3.13	7.51

Most workers place 100 cc. of water in the auto-clave expecting 1 cc. to be lost during sterilization. The results reported give an average loss of 3.2 per cent for water sterilized in bottles carrying cotton plugs. The greatest loss was 4.1 per cent and the least 2.4 per cent.

More water is evidently lost when measured amounts of water are sterilized in the auto-clave than is ordinarily allowed for.

STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

X. SUBGROUPS AND GENERA OF THE MYXOBACTERIALES AND SPIROCHAETALES

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Order V. **Myxobacteriales.** Ordo nov.

Motile, rod-like organisms, multiplying by fission, secreting a gelatinous base, and forming a pseudoplasmodium-like aggregation before passing into a more or less highly developed cyst-producing, resting state in which the rods may become encysted in groups without modification, or may be converted into spore masses.

There is one family only, the *Myxobacteriaceae.*

Family I. **Myxobacteriaceae** Thaxter, 1892, p. 394

Characters those of the order.

The following generic names have been used for organisms of this group.

Polyangium Link, 1795
Stigmatella Berkeley and Curtiss, 1857, p. 70
Chondromyces Berkeley and Curtiss, 1857, p. 313
Cystobacter Schroeter, 1886, p. 170
Myxobacter Thaxter, 1892, p. 403
Myxococcus Thaxter, 1892, p. 403
Myxobotrys Zukal, 1896, p. 346

The genera may be differentiated by the following key:

Key to the genera of Myxobacteriaceae

I. Cells not transformed into coccus-like spores when encysted.
  A. Rods forming free cysts in which they remain unmodified. Cysts various, sessile or borne on a more or less highly developed cystophore.
  Genus I. *Chondromyces*

B. Rods forming large rounded cysts, one or more, free within a gelatinous matrix raised above the substratum..........Genus II. *Polyangium*

II. Rods transformed to form definite, more or less encysted, sessile or stalked masses of coccus-like spores.......................Genus III. *Myxococcus*

Genus I. **Chondromyces** Berkeley and Curtiss, 1857, p. 313

Synonyms:

Stigmatella Berkeley and Curtiss, 1857, p. 70
Polycephalum? Kalch and Cke., p. 22
Cystobacter Schroeter, 1886, p. 170
Myxobotrys Zukal, 1896, p. 346

Rods forming free cysts in which they remain unmodified. Cysts various, sessile or borne on a more or less highly developed cystophore.

The type species is *Chondromyces crocatus* Berkeley and Curtiss.

Genus II. **Polyangium** Link, 1795, p. 65

Synonyms:

Myxobacter Thaxter, 1892, p. 394

Rods forming large rounded cysts, one or more, free within a gelatinous matrix raised above the substratum.

The type species is *Polyangium vitellinum* Link.

Genus III. **Myxococcus** Thaxter, 1892, p. 403

Rods slender, curved, swarming together after a vegetative period to form definite more or less encysted sessile masses of coccus-like spores.

The type species is *Myxococcus rubescens* Thaxter

Order VI. **Spirochaetales.** Ord. nov.

Synonyms:

Spirilloflagellata. Krzysztalowicz and Siedlicki

Protozoan-like in many characters. Cells usually relatively slender flexuous spirals: multiplication of cells apparently by longitudinal division in some types, by transverse division in others, or both.

One family is recognized, *Spirochaetaceae.*

Family I. **Spirochaetaceae** Swellengrebel 1907, p. 581

Synonyms:

Spirochaetoidea Dobell, 1911, p. 536
Spironemaceae Gross, 1912, p. 83

Characters those of the order.

The following generic names have been used for organisms of this group:

Spirochaeta Ehrenberg, 1833, p. 313.
Spirochoeta Dujardin, 1841, p. 209
Spirochaete Cohn, 1872, p. 180
Treponema Schaudinn, 1905, p. 1728
Miscrospironema Stiles & Pfender 1905, p. 936
Spironema Vuillemin, 1905, p. 1567
Borrelia Swellengrebel, 1907, p. 582
Cristispira Gross, 1910, p. 41
Saprospira Gross, 1911, p. 188
Spiroschaudinnia Sambon, 1913, p. 833.

The genera recognized may be differentiated by use of the following key:

Key to the genera of Spirochaetaceae

I. Usually saprophytic, free living in water.
  A. Protoplasm spirally wound around an elastic axis filament. Genus I. *Spirochaeta*
  B. Not as in (A), cross section circular............Genus II. *Saprospira*

II. Usually parasitic.
  A. Possessing a "crest" or ridge. Parasitic in mussels. Genus III. *Cristispira*
  B. Without a crest. Parasitic in warm blooded animals. Genus IV. *Treponema*

Genus I. **Spirochaeta** Ehrenberg, 1833, p. 313

Synonyms:

Spirochaete Cohn, 1872, p. 180
Spirochoeta Dujardin, 1841, p. 209

Slender, spiral cells, living free, usually in water containing hydrogen sulphide, actively motile, flexuous. Flagella unknown. Anaerobic. Protoplasm is spirally wound around a flexible or elastic

axis filament. Volutin granules regularly present in the plasma. No differentiation of exterior. Cell circular in cross section.

The type species is *Spirochaeta plicatilis* Ehrenberg.

Genus II. **Saprospira** Gross, 1911, p. 188

Slender spiral cells living free in salt water, actively motile, flexuous. Cross section circular.

The type species is *Saprospira grandis* Gross.

Genus III. **Cristispira** Gross, 1910, p. 41

Spiral organisms known only from the crystalline style of mussels. The body of the organism is circular in cross section, more or less spirally wound and possessing a longitudinal comb or crest which does not extend quite to the tips.

The type species is *Cristispira veneris* Gross.

Genus IV. **Treponema** Schaudinn, 1905, p. 1728

Synonyms:

Spirochaeta of many authors
Spirochaete of many authors
Spironema Vuillemin, 1905, p. not *Spironema* Meek, 1864
Microspironema Stiles & Pfender, December 2, 1905, p. 936
Borrelia Swellengrebel, 1907, p. 582.
Spiroschaudinnia Sambon, 1907, p. 833

Cells slender, spiral, not flattened, attenuated at tips, without crest. Multiplication by longitudinal or by cross division. Parasites in warm blooded animals. Motile.

The type species is *Treponema pallidum* Schaudinn.

REFERENCES

Berkeley, M. J. 1857 Introduction to Cryptogamic Botany.

Berkeley, M. J. 1874 Notes on North American Fungi Grevillea, **3**, 64.

Cohn, Ferd. 1872 Untersuchungen über Bakterien. Beitr. z. Biologie der Pflanzen. **1** (Heft. 1): 127–224.

Dobell, C. Clifford 1911 On Cristispera veneris nov. sp. and the Affinities and Classification of Spirochaetes. Quart. J. Mic. Sc. **56**, 507–540.

DUJARDIN, F. 1841 Historie naturelle des Zoophytes, Paris.

EHRENBERG, C. G. 1833 Dritter Beitrag zur Erkenntniss grosser Organisation in der Richtung des kleinster Raumes. Abhandl. Berl. Akad., pp. 145–336.

GROSS, J. 1910 Cristispira nov. gen. Ein Beitrag zur Spirochätenfrage. Mittheil. zool. Stat. Neapel., **20**, 41–93.

GROSS, J. 1911 Ueber freilebende Spironemaceen. Mittheil. zool. Stat. Neapel., **21**, 188–204.

GROSS, J. 1912 Ueber Systematik, Struktur und Fortpflanzung der Spironemacea. Centralbl. f. Bakteriol. Abt. 1, **65**, 83.

KRZYSZTALOWICZ AND SIEDLICKI 1908 Etude experimentale de la Syphilis Morphologie de *Spirochaeta pallida*. Bull. Acad. Sci. Cracovie. p. 173.

LINK, H. 1795 Dissertationes botanicae. Sverini.

SAMBON, L. 1907 In Manson's Tropical Diseases. p. 83.

SCHAUDINN, F. 1905 "Korrespondenzen" Deutsche med. Wchnschr. **31**, 1728.

SCHROETER, J. 1886 Die Pilze, in Conn's Kryptogamen Flora von Schleisen.

STILES, C. W., AND PFENDER, C. A. 1905 The generic name of the parasite of syphilis. Am. Med. **10**, 936. Dec. 2.

SWELLENGREBEL, M. 1907 Sur la cytologie comparee des Spirochetes. Ann. de l'Inst. Pasteur. **21**, 448 and 562.

THAXTER, ROLAND 1892 On the Myxobacteriaceae, a new order of Schizomycetes. Bot. Gaz. **17**, 394.

VUILLEMIN, PAUL 1905 Sur la denomination de l'agent de la syphilis. Compt. rend. Acad. d. sc. Par. **140**.

ZUKAL, H. 1896 Myxobotrys variabilis Zuk. als Reprasentant einer neuen Myxomyceten Ordnung, Ber. d. deutsch. Bot. Gesellsch. **14**, 346.

A NOTE ON THE NATURE OF THE REACTION OF B. COLI ON ENDO MEDIUM

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There appears to exist much difference of opinion as to the factor or factors which enter into the production of typical colonies of *B. coli* on the Endo plate. Endo (1904), who developed this medium for the differentiation of *B. typhosus* from *B. coli*, states that the red colonies are due to acid produced by the *B. coli*.

Harding and Ostenberg (1912) claim that the red colonies of these organisms on the Endo plate are due to aldehyd formation, and that the gradual disappearance of the color after the first twenty-four to forty-eight hours incubation, is due to oxidation of aldehyd to acid. According to these workers, acid decolorizes Endo medium. De Bord (1917), on the other hand, claims that acid is essential for the production of red colonies; that aldehyds will not bring out the red color in the Endo medium, but acid and aldehyd will. This worker, like Levine, Weldin, and Johnson (1917) speaks of the Endo reaction as the "fuchsin-aldehyd reaction," insisting however that acid is essential for this reaction.

According to Robinson and Rettger (1916), organic acid, especially lactic acid, is the cause of the reddening of colon colonies on Endo medium, and they maintain that the later decolorization of the colonies, is due to alkali formation by the bacteria. These investigators find that a drop of lactic acid added to an Endo plate, produces a shade of red somewhat similar to that produced by *B. coli*. On the other hand, a drop of neutral

formaldehyd added to the Endo medium produces a purple-violet color.

The underlying cause of the different views expressed by these investigators, appears to be due to the variation in the strength of the reagents employed in their respective experiments. It has been observed again and again that fuchsin decolorized with sodium sulfite will behave differently toward different dilutions of the same reagent. The behavior of the fuchsin sodium sulfite solution toward strong and weak acid, or strong and weak aldehyd is such that it can not be compared. Furthermore, the quantity of sodium sulfite employed in the decolorization of the fuchsin also appears to play an important part in such experiments.

The fuchsin-sulfite combination is extremely unstable. Workers have long learned not to expect complete decolorization of the fuchsin with sodium sulfite in hot solutions, because of the dissociation under these conditions. When an Endo plate is exposed to air, the color of the medium becomes pink, probably because the sulfite in the presence of air is oxidized to sulfate, causing the fuchsin color to reappear in part. Very dilute acids also bring out the color to some extent, possibly because of the high degree of dissociation that exists in the mixture.

If inorganic acids of moderate strength be added to fuchsin decolorized with sodium sulfite, decolorization becomes even more complete. The sodium probably combines with the acid to form a salt, liberating sulfur dioxide or sulfurous acid, and causing further decolorization. Concentrated acids will reduce the color of basic fuchsin without the presence of sodium sulfite.

Organic acid added to decolorized fuchsin will cause a reappearance of the color, due possibly to a stronger affinity of basic fuchsin for the organic acid than for the inorganic sulfite, the result being the formation of acid fuchsin.

It might be said in this connection that we are dealing here with an extremely complex organic combination, and it is questionable to what extent one is permitted to draw a conclusion from a simple test tube experiment. The following few observations will be recorded with the hope that they may throw

some light on the original problem, of the cause of the red colonies on the Endo plate.

When strong aldehyd is added to decolorized fuchsin, a strong purple color is produced. Weak aldehyd produces a red color; weak aldehyd in an acid solution, a strong cherry red color. If a proper combination of aldehyd and acid be added to the decolorized fuchsin in a test tube, a metallic film will appear on the surface in a few minutes. This film, however, is not permanent, disappearing after a few hours. Acetone and alcohol in faintly acid solutions will also bring back the color to decolorized fuchsin. This, it might be added, is a matter of text-book knowledge.

It was further observed that, if glucose be substituted for lactose in Endo medium, not only are typically metallic colonies produced by the colon group, but by the typhoid-dysentery group as well. The same was also found to be true when the triatomic alcohol, glycerol, and the hexatomic alcohol, mannitol, were substituted for lactose in Endo medium.

Finally, the following experiments were performed. Two 1000 cc. Erlenmeyer flasks containing 300 cc. of 1 per cent lactose broth, and 1 per cent glycerol broth respectively, were inoculated with a fresh agar slant growth of *B. coli-communior* obtained from feces. These flasks were employed in order to permit large surface exposure. After twenty-four hours' incubation the contents of the flasks were rendered alkaline with sodium carbonate to hold back the organic acids, and distilled.

When the distillates were added to decolorized fuchsin, no change could be observed. When, however, the distillates were rendered faintly acid with acetic acid and added to decolorized fuchsin, the appearance of the color was far more marked than when the same concentration of acetic acid alone was employed. This would indicate that another substance, or possibly other substances, besides acid, are formed, when *B. coli* is grown under partial anaerobic conditions in lactose or glycerol medium, which help to bring back the color to decolorized fuchsin.

In this connection, it might be well to review briefly the chemical nature of some of the substances under discussion.

(1) The close relation which exists between alcohol, aldehyd, and acid is well illustrated by the following equations. If we take ethyl alcohol as our specific example, we have:

1. $CH_3CH_2OH + O = CH_3.CHO + H_2O$
   Ethyl alcohol — Acetaldehyd.
2. $CH_3CHO + O = CH_3.COOH$
   Acetic acid.

Thus, by oxidation, Alcohol → Aldehyd → Acid
and inversely by reduction Acid → Aldehyd → Alcohol.

It is generally believed that oxygen from the air can not effect the oxidation from alcohol to acid except through the agency of "catalyzers" or ferments.

(2) The close relation which exists between aldehydes and ketones can be seen from the following:

The oxidation of primary alcohols yields aldehydes, and the oxidation of secondary alcohols yields ketones—thus

$$CH_3.CHOH.CH_3 + O = CH_3.CO.CH_3 + H_2O$$

Secondary propyl alcohol Dimethyl ketone or acetone.

(3) From a chemical standpoint, the carbohydrates are aldehyd or ketone derivatives of polyhydric alcohols. This is illustrated by glancing at the structural formula of a typical sugar, glucose, and the two alcohols, mannitol and glycerol.

1. Glucose $= CH_2(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CHO$
2. Mannitol $= CH_2(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CH(OH)\ CH_2OH$
3. Glycerol $= CH_2(OH)\ CH(OH)\ CH_2(OH)$

(4) The production of lactic acid from lactose by a number of organisms, including *B. coli*, has led some investigators to the opinion that the reddening of colonies produced by *B. coli* on Endo medium is due to lactic acid. It is indeed likely that the formation of lactic acid is an important intermediary step in the production of typically metallic colonies on Endo agar. However, when the alcohol, glycerol, is substituted for lactose in

Endo medium, the explanation of typical colonies produced on this medium becomes more complex. It is likely that the glycerol is first oxidized into glyceric aldehyd,

$$\underset{\text{Glycerol}}{CH_2(OH)CH(OH)CH_2(OH)} + \dot{O} = \underset{\text{Glyceric aldehyd.}}{CH_2(OH)CH(OH)CHO} + H_2O$$

and this in turn is converted into lactic acid. It will be recalled that Embden and his co-workers (Hammarsten, 1914) have shown that in the formation of lactic acid from glucose by enzymes, glyceric aldehyd is one of the intermediary products.

Grey (1913), who has investigated the products of anaerobic glucose decomposition by *B. coli-communis*, finds that this organism produces lactic, acetic, formic, and succinic acids, alcohol, and acetaldehyd from this carbohydrate. Mendel (1911) finds also the presence of acetone among glucose decomposition products of *B. coli*.

To recapitulate the observations discussed above:

1. Acid, aldehyd, acetone and alcohol in proper dilution and combination cause a reappearance of the fuchsin color of a decolorized fuchsin-sulfite solution.

2. When glucose, mannitol and glycerol are substituted for lactose in the Endo medium, typically metallic colonies are produced by the entire typhoid-colon group.

3. *B. coli-communior*, when grown in lactose and glycerol broth under partially anaerobic conditions, produces another substance—or possibly substances—besides acid, which bring back the color to decolorized fuchsin, when faintly acidified.

In view of these observations and those of the workers mentioned above, it would appear that the red colonies which *B. coli* produces on the Endo plate are due to a number of substances—including lactic and other organic acids, traces of aldehyd and possibly also acetone and alcohol. The latter of these substances are volatile and therefore evaporate on exposure, the result being that the non-volatile fuchsin remains behind and we thus have the metallic film.

The possible criticism that *B. coli* breaks down glucose into the various substances named above, only when grown anaerobi-

cally, but not during the aerobic growth on Endo agar, does not appear to hold. Organisms growing on the surface of agar must indeed have the ability to grow in the presence of air in order to get a start, so to speak. But once the start is gained the surface organisms alone grow aerobically, while those organisms below the surface of the colony are probably growing in no less an anaerobic environment than those growing below the surface in carbohydrate broth.

Observations of the gradual disappearance of the metallic film of colon colonies on Endo agar corroborates this view. It is reasonable to assume that the organisms below the metallic film of a colon colony, are growing in an anaerobic environment. These organisms, after twenty-four to forty-eight hours incubation, proceed to break down the nitrogenous bodies of the nutrient media into ammonia and amines (Robinson and Rettger), creating a condition favorable for the re-solution of the metallic fuchsin and final decolorization. In other words, we have here a chemical change produced by *B. coli* growing *anaerobically* in apparently aerobic colonies.

As further evidence that the metallic sheen of colon colonies on Endo medium results from the evaporation of volatile substances produced during the metabolism of these organisms, the following two observations are cited.

1. If *colon bacilli* are grown anaerobically in Endo agar, the colonies are red but not metallic. Under anaerobic conditions, *B. coli* breaks down carbohydrate into various acids, aldehyd, and possibly acetone and alcohol (Grey and Mendel). These substances bring out the color of decolorized fuchsin and the colonies become red. The fuchsin, however, remains in solution, because the volatile substances can not evaporate. The same colonies exposed to air become metallic, because surface evaporation permits the disappearance of the substances which hold the fuchsin in solution.

2. The metallic sheen of a given colon colony is markedly pronounced when grown in such a manner that evaporation can take place on a large scale. Thus, if *B. coli* be inoculated on Endo plates and on Endo agar slants, the metallic film, after a

given period of incubation, will be more pronounced on the Endo agar slant than on the Endo plate. This is explained as due to the greater opportunity for evaporation from the slant han from the inverted plate.

There are a large number of red colonies on Endo medium which workers recognize to be of non-colon types, These colonies, as a rule, possess a red shade that is somewhat different from that of *B. coli* and they never possess a metallic film. It is possible that some organisms cause the production of red colonies by reducing sodium sulfite to hydrogen sulfide, thus liberating the fuchsin color. It is more likely, however, in view of the ease with which bacteria attack carbohydrates, that these red colonies are produced by the conversion of lactose to some non-volatile acid, most probably lactic acid. This acid has a tendency to absorb moisture from the air, and such moisture on the surface of the colony would tend to keep the fuchsin in solution, rendering the colony red but not metallic.

CONCLUSIONS

1. Chemical and physical factors enter into the formation of typically metallic colonies of *B. coli* on Endo agar.
2. During the growth of these organisms on Endo medium, they adsorb the various soluble substances contained in the medium and proceed to break down the lactose first (Kendall).
3. After ten to fifteen hours incubation, the trace of lactose adsorbed, is probably transformed into lactic and other organic acids, and the colony is colored red.
4. On further incubation, the organic acids are probably reduced by the bacteria to aldehyd and alcohol, which volatilize from the surface, leaving the non-volatile fuchsin behind, thus producing a metallic sheen.
5. The carbohydrate being disposed of, the organisms proceed to attack the nitrogenous materials. Ammonia and other substances are produced in which the fuchsin goes back into solution, and ultimate decolorization takes place.

REFERENCES

DeBord 1917 J. Bact., **2**, 309.
Endo 1904 Centralbl. f. Bakteriol. Orig. I, **35**, 109.
Grey 1913 Bio-Chem. J., **7**, 359.
Hammersten 1914 Textbook of Physiological Chemistry, p. 333.
Harding and Ostenberg 1912 J. Infect. Dis., **11**, 109.
Levine, Weldin and Johnson 1917 J. Infect. Dis., **21**, 39.
Mendel 1911 Centralbl. f. Bakteriol. II, **29**, 290.
Robinson and Rettger 1916 J. Med. Research, **29**, 363.

A SIMPLIFIED CONFIRMATORY TEST FOR B. COLI

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When the presumptive test for *B. coli* has been established by gas production in lactose broth, we have, according to the committee on Standard Methods of Water Analysis (1917), two more steps to carry out before the presence of *B. coli* is completely confirmed. Transplantations are made from the lactose fermentation tubes showing gas, to Endo plates and typical colonies sought for after twenty-four hours incubation. If such colonies be found, the test for *B. coli* is "partially confirmed." One or more of these colonies are again inoculated in lactose fermentation tubes and examined for gas production after incubation. The presence of gas in these fermentation tubes renders the presence of *B. coli* completely established.

During our investigation of the nature of the reaction of *B. coli* on Endo medium (1918), it was observed that colonies of *B. coli* are more typically metallic when grown on Endo agar slants than on inverted Endo plates. This finding has led to the view that possibly test tube slants containing Endo medium, might advantageously be substituted for Endo plates in the confirmatory test for *B. coli* in water.

It appeared also that if the inoculation of these organisms in an Endo tube be made both in the butt and on the slant, we would have, after proper incubation, typically metallic colonies on the slant and a practically anaerobic condition in the butt with gas production and reddening of the medium. We should thus have the "partially confirmed" and "completed" tests for *B. coli* in one procedure.

When testing out the growth of a number of strains of *B. coli* under these conditions, it was observed that while the colonies on the surface of the slant were typically metallic, the organisms growing in the butt, although showing gas production, did not always redden the medium during the first twenty-four hours of incubation. This fact, however, was not found to interfere with our intention of utilizing the Endo tube rather than the plate for the confirmatory test for *B. coli* in water. When waters sent to this laboratory for examination showed gas formation in lactose broth fermentation tubes, straight wire inoculations were made from these tubes, to Endo tubes, extending the wire first into the butt, then spreading the tip of the wire over the slant. Endo plates were also inoculated as a check. The results were always constant. When colonies of *B. coli* were present on the Endo plates, typical colonies were present also on the slant, with gas production in the butt. Occasionally, after a given period in the incubator, the colonies on the plate would require further incubation, while those on the slant would be sufficiently metallic to render the test complete.

This constancy of results, combined with the saving of time and material by this new procedure, led to the complete substitution of Endo agar slants for Endo plates in the confirmatory test for *B. coli*.

The following procedure also, has been employed as a confirmatory test for *B. coli:* A loop from the lactose fermentation tube showing gas, is spread over the surface of an empty Petri dish. A tube of Endo agar is now melted in a water bath, cooled to 40°C. and poured over the surface of the dish. When the Endo agar is congealed, a loop from the same fermentation tube is smeared over the surface of the agar. After incubation, if *B. coli* be present, typical colonies will appear on the surface and red colonies with gas bubbles, below the surface of the agar. Thus an aerobic growth and a practically anaerobic growth is obtained in one test.

However, no marked advantage is gained when employing this procedure over the simple Endo tube method described above.

Occasionally *B. coli* are found in water which form non-typical colonies on Endo medium, i.e., colonies without a metallic sheen. Under such conditions, at least two colonies considered to be most likely *B. coli*, are transferred into lactose broth fermentation tubes (Standard Method) and gas formation observed after twenty-four and forty-eight hours. The presence of gas in these tubes constitutes a positive test, while the absence of gas at the end of forty-eight hours constitutes a negative test for *B. coli*. In view of the difficulty in differentiating non-typical colonies of *B. coli* from non-colon colonies on the Endo plate, it is thus possible, by inoculating non-colon colonies into fermentation tubes, to miss the detection of *B. coli*. On the other hand, when such colonies are grown in Endo tubes, we have, aside from their growth on the slant, also gas formation in the butt. We thus have two factors which help to establish the presence of the organisms.

There is, of course, a remote possibility that we might have colon-like colonies on an Endo agar slant and gas formation in the butt, due to an anaerobe instead of *B. coli*. When such a condition is suspected, inoculations of several colon-like colonies in lactose broth fermentation tubes, should be resorted to. It might be mentioned however, that in our work with anaerobic lactose fermenting organisms, obtained from waters of the southeastern area of the United States, in not a single instance did such organisms produce gas in the butt of Endo tubes.

Another important advantage of the substitution of the Endo tube for the Endo plate in confirmatory tests for *B. coli*, lies in the fact that tubes can be kept in the ice-box from three to four weeks without deterioration. Russell (1912) and Robinson and Rettger (1916) have suggested that Endo medium for typhoid work be first stored in tubes, and plates poured from these when ready for use. These investigators found that this medium keeps well in tubes from two to three weeks in the ice-box.

It is well known that Endo plates, even if kept in the dark, will begin to redden after forty-eight hours. This appears to be due to the large surface exposed to air, which brings about the oxidation of the unstable sodium sulfite to sulfate. Tubes con-

taining Endo medium were kept on the working table exposed to direct light for close to two weeks without any appreciable change in the butt; the slant, however, became red after twenty-four hours. This is explained by the fact that air can not come in direct contact with the medium in the butt; no oxidation of the sulfite takes place, and the medium remains unchanged. Endo tubes have been kept in this laboratory for four weeks in the ice-box without any apparent deterioration.

It might also be added that unless a sufficient number of previous examinations of a given water have established the absence of *B. coli* in it, we do not wait for gas production in lactose broth before inoculating in Endo tubes. It was observed again and again that if waters showing a high bacterial count be inoculated in lactose broth fermentation tubes and after about fifteen hours incubation—when there is no sign of gas production—inoculations made in Endo tubes, typically metallic colonies on the Endo slant with gas production in the butt often develop simultaneously with gas in the original lactose fermentation tubes. Thus, at the appearance of the "presumptive test" we would have the "completed test" also. These findings have led to the application of this procedure to all waters coming into this laboratory in which the absence of *B. coli* has not been established by previous tests.

The question of the strength of agar to be employed in the Endo tube media is of some importance. In a comparative study of Endo media containing 1.5 per cent and 3 per cent agar, no marked variations were observed in the amount of gas produced in the butt. A strong gas producing organism would usually produce much gas, while a weak one, would produce only a few bubbles, in both cases.

It was observed also that colonies of *B. coli* have, as a rule, a stronger metallic film, when grown on Endo medium containing 1.5 per cent agar than 3 per cent agar. The difficulty with the former, however, is the fact that the surface of the slant is apt to be moist. This moisture may prevent the formation of isolated colonies of *B. coli*—and such colonies are desirable for the final isolation of these organisms.

Of late we have been employing Endo medium of 2 per cent agar, with good results. The surface of the slant is, as a rule, dry and there is no difficulty in obtaining isolated colonies.

There also exists some difference of opinion as to the amount of fuchsin to be employed in Endo medium for colon work. In this laboratory, 0.2 per cent of a saturated alcoholic solution of basic fuchsin in agar is employed for the isolation of *B. typhosus* from feces. This amount of fuchsin approaches that suggested in the "Standard Methods," and although sufficient for typhoid work, is too weak for the detection of *B. coli* in water. Hasseltine (1917) comes to the same conclusion. 0.5 cc. and 0.4 cc. of a saturated alcoholic solution of basic fuchsin per 100 cc. of agar have been employed with good results. Our findings would indicate that 0.4 per cent of fuchsin is sufficient for colon work.

The method of sterilization suggested by Robinson and Rettger (1916) has been employed for this medium. Stock quantities of 2 per cent agar, titrated to 0.2 to phenolphthalein are kept on hand. Just before tubing, proper quantities of sterile lactose, fuchsin, and fresh sodium sulfite are added to the agar previously melted in the Arnold. The tubing is carried out with care to prevent undue contamination. The tubes are then placed in the hot autoclave and sterilized from five to seven minutes at 10 pounds pressure. After sterilization, the tubes are slanted and covered with a towel to keep out the light, after which they are placed in the ice-box and are ready for use.

Whether or not typically metallic colonies are produced on Endo medium by organisms not related to the colon group has been under investigation for some time. When glycerol or glucose is substituted for lactose in Endo medium, other organisms beside *B. coli*, produce red colonies with a metallic film. When, however, regular Endo medium is employed with lactose as the carbohydrate, no metallic colonies were observed except by members of the colon group.

Fifty Endo plates were exposed to air and were then incubated; twenty-five plates at 37°C., and twenty-five plates at room temperature. Observations were made every day for two

combination is sufficiently toxic to prevent their growth even in the butt.

This lack of growth in Endo tubes after inoculations from lactose broth fermentation tubes showing gas, has helped us to differentiate anaerobic lactose fermenting organisms from *B. coli*, in a large number of cases.

Frequently, however, when gas production in lactose broth is due to an anaerobe, other organisms capable of aerobic growth are found in the medium. Under these conditions, after transplantations from fermentation tubes to Endo tubes, a growth would occasionally appear on the slant, but as a rule, would be recognized without difficulty to be of non-colon type.

As a further means for differentiating lactose fermenting anaerobes from *B. coli* the following procedure may be employed:

A loop from the lactose fermentation tube showing gas, is smeared on the bottom of a Petri dish. A tube of Endo agar is melted in a water bath, cooled to about 40°C. and poured over the surface of the plate. After the Endo agar is congealed, another loop from the same fermentation tube is now spread over the surface of the agar. We thus have, after incubation, an aerobic growth on the surface and a practically anaerobic growth below the surface of the Endo agar.

If *B. coli* be present, typical or semi-typical colonies will appear on the surface; also red colonies, and as a rule, gas bubbles will appear below the surface of the medium. If on the other hand, the gas produced in lactose broth is due to an anaerobe, typical colon colonies are of course, never present; the colonies below the surface of the agar are, as a rule, uncolored; neither are gas bubbles observed below the surface of the medium.

It is well to keep in mind that while *B. coli* represent a definite group of organisms with well defined characteristics, the anaerobes found in water vary widely both in traits and morphology. It is likely that geographic and climatic conditions will favor the growth of certain types of anaerobes in one area, and of different types of anaerobes in another area of the country. A procedure, therefore, which would help differentiate anaerobes from *B.*

coli, applied for waters of the southeastern area of the United States, may not be applicable to waters of the northern and western areas of the country. Thus the anaerobes which we have been able to observe, have not in a single instance produced gas in the butt of Endo tubes. On the other hand, it is likely that anaerobes found in waters in other parts of the country might possess this ability.

The ideal procedure for the differentiation of anaerobic lactose fermenting organisms from *B. coli* should not only establish the absence of *B. coli*, but permit the isolation of the anaerobe as well. Simonds and Kendall (1912) and Jones (1916) have described good procedures for the isolation of anaerobes in pure culture. The application of these methods to the isolation of anaerobes found in water, would provide valuable scientific data. The practical worker, however, would probably find the isolation of anaerobes by either of these methods not commensurable with the work involved, since the mere isolation of a lactose fermenting anaerobe would not rule out the possible presence of *B. coli*, and it is this fact that the worker is primarily interested in.

It might be added that it is questionable whether "anaerobes" is a proper term to apply to non-colon lactose fermenting organisms found in water. We have twice transplanted spreading growths from Endo plates into lactose fermentation tubes which resulted in gas formation in these tubes after incubation. These growths on the Endo plates could readily be distinguished as non-colon types. It is evident, however, that not all non-colon lactose fermenters, are anaerobes. Meyer (1918) has recently reported the finding of a lactose fermenting organism in water, capable of growing aerobically.

RESUMÉ

1. Anaerobic lactose fermenting organisms found in waters of the Southeastern area of the United States, appear to be unable to produce gas when grown anaerobically in Endo medium. In accordance with this finding, the differentiation of anaerobes from *B. coli*, is accomplished as follows:

2. Straight wire inoculations are made from the lactose fermentation tube showing gas, into the butt and on the slant of Endo tubes. After incubation (1) *B. coli* produce typical or semi-typical colonies on the slant and gas in the butt. (2) Anaerobes neither grow on the slant nor produce gas in the butt of these tubes.

3. Employing the same medium, another procedure for the differentiation of anaerobes is described in the text, wherein plates are employed instead of tubes.

REFERENCES

Committee on Standard Methods of Water Analysis 1917 American Public Health Association, Boston.

Frost 1916 Am. J. Pub. Health., **6**, 585.

Jones 1916 J. Bact., **1**, 339.

Meyer 1918 J. Bact., **3**, 9.

Simonds and Kendall 1912 J. Inject. Dis., **11**, 207.

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